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**(54) Title:** NEURTURIN RECEPTOR**(57) Abstract**

NTNR $\alpha$ , NTNR $\alpha$  extracellular domain (ECD), NTNR $\alpha$  variants, chimeric NTNR $\alpha$  (e.g., NTNR $\alpha$  immunoadhesion), and antibodies which bind thereto (including agonist and neutralizing antibodies) are disclosed. Various uses for these molecules are described, including methods to modulate cell activity and survival by response to NTNR $\alpha$ -ligands, for example NTN, by providing NTNR $\alpha$  to the cell.

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## NEURTURIN RECEPTOR

### INTRODUCTION

#### Technical Field

The present invention relates to a Neurturin ("NTN") receptor designated NTNR $\alpha$  (also referred to as GFR $\alpha$ 2), and provides for NTNR $\alpha$ -encoding nucleic acid and amino acid sequences. In particular, the invention relates to native sequence NTNR $\alpha$ , NTNR $\alpha$  variants, soluble NTNR $\alpha$  variants including NTNR $\alpha$  extracellular domain, chimeric NTNR $\alpha$ , and antibodies which bind to the NTNR $\alpha$  (including agonist and neutralizing antibodies), as well as various uses for these molecules. It also relates to assay systems for detecting ligands to NTNR $\alpha$ , systems for studying the physiological role of NTN, diagnostic techniques for identifying NTN-related conditions, therapeutic techniques for the treatment of NTN-related and NTNR $\alpha$ -related conditions, and methods for identifying molecules homologous to NTNR $\alpha$ .

#### Background

Neurotrophic factors such as insulin-like growth factors, nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, -4/5 and -6, ciliary neurotrophic factor, GDNF, and neurturin have been proposed as potential means for enhancing specific neuronal cell survival, for example, as a treatment for neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer's disease, stroke, epilepsy, Huntington's disease, Parkinson's disease, and peripheral neuropathy. It would be desirable to provide additional therapy for this purpose. Protein neurotrophic factors, or neurotrophins, which influence growth and development of the vertebrate nervous system, are believed to play an important role in promoting the differentiation, survival, and function of diverse groups of neurons in the brain and periphery. Neurotrophic factors are believed to have important signaling functions in neural tissues, based in part upon the precedent established with nerve growth factor (NGF). NGF supports the survival of sympathetic, sensory, and basal forebrain neurons both *in vitro* and *in vivo*. Administration of exogenous NGF rescues neurons from cell death during development. Conversely, removal or sequestration of endogenous NGF by administration of anti-NGF antibodies promotes such cell death (Heumann, *J. Exp. Biol.*, 132:133-150 (1987); Hefti, *J. Neurosci.*, 6:2155-2162 (1986); Thoenen *et al.*, *Annu. Rev. Physiol.*, 60:284-335 (1980)).

Additional neurotrophic factors related to NGF have since been identified. These include brain-derived neurotrophic factor (BDNF) (Leibrock, *et al.*, *Nature*, 341:149-152 (1989)), neurotrophin-3 (NT-3) (Kaisho, *et al.*, *FEBS Lett.*, 266:187 (1990); Maisonpierre, *et al.*, *Science*, 247:1446 (1990); Rosenthal, *et al.*, *Neuron*, 4:767 (1990)), and neurotrophin 4/5 (NT-4/5) (Berkmeier, *et al.*, *Neuron*, 7:857-866 (1991)).

Neurotrophins, similar to other polypeptide growth factors, affect their target cells through interactions with cell surface receptors. According to current understanding, two kinds of transmembrane glycoproteins act as receptors for the known neurotrophins. Equilibrium binding studies have shown that neurotrophin-responsive neuronal cells possess a common low molecular weight (65,000 - 80,000 Daltons), a low affinity receptor typically referred to as p75<sup>LNGFR</sup> or p75, and a high molecular weight (130,000-150,000 Dalton) receptor. The high affinity receptors are members of the trk family of receptor tyrosine kinases.

Receptor tyrosine kinases are known to serve as receptors for a variety of protein factors that promote cellular proliferation, differentiation, and survival. In addition to the trk receptors, examples of other receptor

tyrosine kinases include the receptors for epidermal growth factor (EGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF). Typically, these receptors span the cell membrane, with one portion of the receptor being intracellular and in contact with the cytoplasm, and another portion of the receptor being extracellular. Binding of a ligand to the extracellular portion of the receptor induces tyrosine kinase activity in the intracellular portion of the receptor, with ensuing phosphorylation of various intracellular proteins involved in cellular signaling pathways.

Glial cell line-derived neurotrophic factor ("GDNF") and Neurturin ("NTN") are two, recently identified, structurally related, potent survival factors for sympathetic sensory and central nervous system neurons (Lin *et al. Science* 260:1130-1132 (1993); Henderson *et al. Science* 266:1062-1064 (1994); Buj-Bello *et al., Neuron* 15:821-828 (1995); Kotzbauer *et al. Nature* 384:467-470 (1996)). Recently, GDNF was shown to mediate its actions through a multi-component receptor system composed of a ligand binding glycosyl-phosphatidyl inositol (GPI) linked protein (designated GDNFR $\alpha$ ; also designated GFR- $\alpha$ -1) and the transmembrane receptor tyrosine kinase Ret (Treanor *et al. Nature* 382:80-83 (1996); Jing *et al. Cell* 85:1113-1124 (1996); Trupp *et al. Nature* 381:785-789 (1996); Durbec *et al. Nature* 381:789-793 (1996)). The mechanism by which the NTN signal is transmitted has not been elucidated.

Aberrant expression of receptor tyrosine kinases ("RTK") correlates with transforming ability. For example, carcinomas of the liver, lung, breast and colon show elevated expression of Eph RTK. Unlike many other tyrosine kinases, this elevated expression can occur in the absence of gene amplification or rearrangement. Moreover, Hek, a human RTK, has been identified as a leukemia-specific marker present on the surface of a pre-B cell leukemia cell line. As with Eph, Hek also was overexpressed in the absence of gene amplification or rearrangements in, for example, hemopoietic tumors and lymphoid tumor cell lines. Over-expression of Myk-1 (a murine homolog of human Htk (Bennett *et al., J. Biol. Chem.*, 269(19):14211-8 (1994)) was found in the undifferentiated and invasive mammary tumors of transgenic mice expressing the Ha-ras oncogene. (Andres *et al., Oncogene*, 9(5):1461-7 (1994) and Andres *et al., Oncogene*, 9(8):2431 (1994)). Ret, the product of the c-ret proto-oncogene, is a member of the receptor tyrosine kinase superfamily.

In addition to their roles in carcinogenesis, a number of transmembrane tyrosine kinases have been reported to play key roles during development. Some receptor tyrosine kinases are developmentally regulated and predominantly expressed in embryonic tissues. Examples include Cek1, which belongs to the FGF subclass, and the Cek4 and Cek5 tyrosine kinases (Pasquale *et al., Proc. Natl. Acad. Sci., USA*, 86:5449-5453 (1989); Sajjadi *et al., New Biol.*, 3(8):769-78 (1991); and Pasquale, *Cell Regulation*, 2:523-534 (1991)). Eph family members are expressed in many different adult tissues, with several family members expressed in the nervous system or specifically in neurons (Maisonpierre *et al., Oncogene*, 8:3277-3288 (1993); Lai *et al., Neuron*, 6:691-704 (1991)).

The aberrant expression or uncontrolled regulation of any one of these receptor tyrosine kinases can result in different malignancies and pathological disorders. Therefore, there exists a need to identify means to regulate, control and manipulate receptor tyrosine kinases ("RTK") and their associated ligands or GPI-linked receptors, in order to provide new and additional means for the diagnosis and therapy of receptor tyrosine kinase pathway-related disorders and cellular processes. The present application provides the clinician and researcher with such means by providing new molecules that are specific for interacting with

certain RTK receptors. These compounds and their methods of use, as provided herein, allow exquisite therapeutic control and specificity. Accordingly, it is an object of the present invention to provide an improved therapy for the prevention and/or treatment of neurological conditions and other conditions in which certain neurotrophic signaling pathways play a role.

5        These and other objects of the invention will be apparent to the ordinarily skilled artisan upon consideration of the specification as a whole.

#### SUMMARY

10        A NTN receptor termed NTN $\alpha$ , a soluble form of the receptor, and a NTN $\alpha$  extracellular domain ("ECD") are disclosed herein. Also disclosed are NTN $\alpha$  polypeptides, optionally conjugated with or fused to molecules which increase the serum half-lives thereof, and optionally formulated as pharmaceutical compositions with a physiologically acceptable carrier.

15        Soluble NTN $\alpha$ , including chimeric NTN $\alpha$  molecules such as NTN $\alpha$  ECD immunoadhesins (having long serum half-lives) and epitope-tagged NTN $\alpha$  ECD, that retain both ligand binding, preferably NTN binding, and receptor signaling function (via Ret receptor tyrosine kinase) can be used to impart, restore, or enhance NTN $\alpha$ -ligand (preferably NTN) responsiveness to cells. This responsiveness includes ligand-binding, Ret tyrosine phosphorylation and Ret-mediated downstream activity, which can result in modulation of cell activity such as survival or growth. The embodiments find use *in vivo*, *in vitro* or *ex vivo*. Soluble NTN $\alpha$  forms that bind NTN but fail to interact with and activate Ret can be used as an antagonist to NTN ligand (by binding and sequestering NTN) to reduce activation of endogenous NTN $\alpha$ . This is useful in conditions characterized by excess levels of NTN ligand and/or excess NTN $\alpha$  activation in a mammal. Bispecific immunoadhesins (for example, combining a NTN $\alpha$ -ligand binding activity with a ligand-binding domain of another cytokine or neurotrophic factor receptor) can form high affinity binding complexes for NTN $\alpha$ -ligands and another factors, providing either antagonist activity (when Ret-activating function is absent) or a means to enhance the attached ligands delivery.

25        Pharmaceutical compositions of soluble NTN $\alpha$ , preferably ECD, can optionally further include an NTN $\alpha$  ligand, preferably NTN. Such compositions are useful where it is desirable to prolong the half-life of the ligand, provide slow, sustained release of ligand, impart NTN $\alpha$ -ligand responsiveness to a target cell, and/or activate or enhance endogenous cellular NTN $\alpha$  or Ret activity directly. Optionally, the composition further contains one or more cytokines, neurotrophic factors, or their agonist antibodies.

30        Also provided are methods for identifying a molecule which binds to and/or activates NTN $\alpha$ . Thus assays are provided to screen for or identify NTN $\alpha$ -ligand molecules (such as peptides, antibodies, and small molecules) that are agonists or antagonists of NTN $\alpha$ . Such methods generally involve exposing an immobilized NTN $\alpha$  to a molecule suspected of binding thereto and determining binding of the molecule to the immobilized NTN $\alpha$  and/or evaluating whether or not the molecule activates (or blocks activation of) the NTN $\alpha$ . In order to identify such NTN ligands, the NTN $\alpha$  can be expressed on the surface of a cell and used to screen libraries of synthetic candidate compounds or naturally-occurring compounds (*e.g.*, from endogenous sources such as serum or cells). NTN $\alpha$  can also be used as a diagnostic tool for measuring serum levels of endogenous or exogenous NTN $\alpha$ -ligand.

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In a further embodiment, a method for purifying an NTNR $\alpha$ -ligand is provided. This finds use in commercial production and purification of therapeutically active molecules that bind to this receptor. In one embodiment the molecule of interest (generally in a composition comprising one or more contaminants) is adsorbed to immobilized NTNR $\alpha$  (e.g., NTNR $\alpha$  immunoadhesin immobilized on a protein A resin). The contaminants, by virtue of their inability to bind to the NTNR $\alpha$ , will generally not bind the resin. Accordingly, it is then possible to recover the molecule of interest from the resin by changing the elution conditions, such that the ligand molecule is released from the immobilized receptor.

Antibodies are provided that specifically bind to NTNR $\alpha$ . Preferred antibodies are monoclonal antibodies that are non-immunogenic in a human and bind to an epitope in the extracellular domain of the receptor. Preferred antibodies bind the NTNR $\alpha$  with an affinity of at least about  $10^6$  L/mole, more preferably  $10^7$  L/mole. Preferred antibodies are agonist antibodies.

Antibodies, which bind to NTNR $\alpha$ , can be optionally fused to a heterologous polypeptide. The antibody or fusion finds particular use to isolate and purify NTNR $\alpha$  from a source of the receptor.

In a further aspect is provided a method for detecting NTNR $\alpha$  *in vitro* or *in vivo* which includes the steps of contacting an NTNR $\alpha$  antibody with a sample suspected of containing the receptor, and detecting if binding has occurred.

For certain applications it is desirable to have an agonist antibody. Such agonist antibodies are useful for activating NTNR $\alpha$  as described for NTNR $\alpha$ -ligands such as NTN. Furthermore, these antibodies are useful to treat conditions in which an effective amount of NTNR $\alpha$  activation leads to a therapeutic benefit in the mammal. For example, the agonist antibody can be used to elicit an NTN response in a cell comprising NTNR $\alpha$  and, preferably, Ret. For therapeutic applications it is desirable to prepare a composition having the agonist antibody and a physiologically acceptable carrier. Optionally, the composition further contains one or more cytokines, neurotrophic factors, or their agonist antibodies.

In other embodiments, the antibody is a neutralizing antibody. Such molecules can be used to treat conditions characterized by unwanted or excessive activation of NTNR $\alpha$ .

In addition to the above, the invention provides isolated nucleic acid molecules, expression vectors and host cells encoding NTNR $\alpha$  which can be used in the recombinant production of NTNR $\alpha$  as described herein. The isolated nucleic acid molecules and vectors are also useful to prepare transgenic animals, for gene therapy applications to treat patients with NTNR $\alpha$  defects or increase responsiveness of cells to NTNR $\alpha$  ligands, or alternatively to decrease NTNR $\alpha$  activity (as by use of antisense nucleic acid).

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1B depict the nucleic acid sequence (SEQ ID NO.: 1) of the sense strand of the cDNA encoding full length human NTNR $\alpha$ , the hNTNR $\alpha$ -encoding sequence (SEQ ID NO.: 2), and the deduced amino acid sequence of full length hNTNR $\alpha$  (SEQ ID NO.: 3). Nucleotides are numbered at the beginning of the sense strand. Amino acid residues are numbered at the beginning of the amino acid sequence.

Figures 2A-2B depict the nucleic acid sequence (SEQ ID NO.: 4) of the sense strand of the cDNA encoding full length rat NTNR $\alpha$ , the rNTNR $\alpha$ -encoding sequence (SEQ ID NO.: 5), and the deduced amino acid sequence of full length rNTNR $\alpha$  (SEQ ID NO.: 6). Nucleotides are numbered at the beginning of the sense strand. Amino acid residues are numbered at the beginning of the amino acid sequence.

Figures 3A-3B compare hNTNR $\alpha$ -, rNTNR $\alpha$ -, and rGDNFR $\alpha$ -encoding nucleic acids.

Figure 4 is a comparison of the hNTNR $\alpha$ , rNTNR $\alpha$ , and rGDNFR $\alpha$  proteins, with features indicated. Signal peptides are indicated by a solid line. Signal cleavage sites are marked with arrows. Potential glycosylation sites are shaded. The hydrophobic domain of the GPI attachment site is doubly underlined. The small amino acid residues that constitute a cleavage/attachment site for GPI-linked proteins are marked with asterisks. Consensus cysteine residues are indicated by a solid circle. The extracellular domain ("ECD") is flanked by the signal peptide and the GPI-attachment site.

Figure 5 is a comparison of the amino acid sequences of hNTNR $\alpha$  and hGDNFR $\alpha$ .

Figures 6A-6D depict binding of  $^{125}$ I NTN and GDNF to NTNR $\alpha$ - or GDNFR $\alpha$ -expressing cells and displacement by unlabeled NTN. Figures 6A and 6C show the binding of  $^{125}$ I mouse NTN ( $^{125}$ I-mNTN) to rat GDNFR $\alpha$  ("rGDNFR $\alpha$ ") or rat NTNR $\alpha$  ("rNTNR $\alpha$ "), respectively. Figures 6B and 6D show the binding of  $^{125}$ I-rGDNF ( $^{125}$ I-rGDNF) to rat GDNFR $\alpha$  ("rGDNFR $\alpha$ ") or rat NTNR $\alpha$  ("rNTNR $\alpha$ "), respectively. As depicted by the Scatchard analysis, displayed in the inset of Fig. 6B, GDNF binds GDNFR $\alpha$  with a  $K_d$  value of 3 pM. A similar  $K_d$  was reported in a cell based assay (Jing *et al. Cell* 85:1113-1124 (1996)). Mouse NTN binds rNTNR $\alpha$  with a  $K_d$  value of 10 pM (see inset to Fig. 6C). Human NTNR $\alpha$  displayed a similar binding specificity as rat NTNR $\alpha$  (data not shown). Although in these experiments no binding of  $^{125}$ I NTN to GDNFR $\alpha$  (Fig. 6A) and  $^{125}$ I-rGDNF to NTNR $\alpha$  (Fig. 6D) were detected, experiments performed with biotinylated NTN and GDNF revealed low affinity binding ( $K_d$  above 1mM) of NTN to GDNFR $\alpha$ , and vice versa.

Figures 7A-7F depict interaction between NTN, NTNR $\alpha$  and Ret. Figure 7A depicts binding of  $^{125}$ I NTN to cells expressing NTNR $\alpha$ . Consistent with the prediction that NTNR $\alpha$  is a GPI-linked protein, binding of  $^{125}$ I NTN to NTNR $\alpha$  expressing cells was reduced by 50-70% following treatment with PIPLC. Figure 7B depicts survival response of embryonic, rat spinal motoneurons to GDNF or NTN. In agreement with its receptor distribution, NTN is a potent survival factor for spinal motoneurons. Figure 7C depicts survival response of embryonic, rat spinal motoneurons to NTN or BDNF in the presence of PIPLC and a soluble NTNR $\alpha$ . PIPLC treatment reduced the survival response to NTN by 50-90% without changing the response to BDNF. Soluble NTNR $\alpha$  (sR $\alpha$ ) restores the response of PIPLC-treated motoneurons to NTN. Figure 7D depicts NTN induction of tyrosine phosphorylation of Ret in neuroblastoma TGW-1 cells. Figure 7E depicts NTN induction of phosphorylation of ERK in TGW-1. Figure 7F depicts the NTN-responsiveness (e.g., Ret phosphorylation) imparted by an NTN-soluble NTNR $\alpha$  complex to Ret-expressing cells. Legends: (Con) = untransfected cells. (Ret) = cells transfected with Ret alone. (R $\alpha$ +Ret) = cells transfected with Ret and NTNR $\alpha$ . In all cases, cells were exposed to NTN (100ng/ml) and then processed for immunoprecipitation with NTN antisera.

Figures 8A to 8C depict the survival response of dopaminergic neurons to soluble Ret-activating forms of NTNR $\alpha$  and GDNFR $\alpha$  extracellular domains.

Figure 9 depicts the ratio of DOPAC to dopamine (expressed as the injected side as a percentage of the uninjected side, mean  $\pm$  sem) in various brain regions, particularly the striatum, from rats injected in one striatum with NTN, a soluble form of NTNR $\alpha$ , both NTN and NTNR $\alpha$ , or vehicle.

## DETAILED DESCRIPTION

In describing the present invention, the following terms will be employed and are intended to be defined as indicated below.

The terms "NTNR $\alpha$ " (also designated GFR- $\alpha$ -2) or "NTNR $\alpha$  polypeptide" when used herein encompass native sequence NTNR $\alpha$ ; NTNR $\alpha$  variants; NTNR $\alpha$  extracellular domain; and chimeric NTNR $\alpha$  (each of which is defined herein). Optionally, the NTNR $\alpha$  is not associated with native glycosylation. "Native glycosylation" refers to the carbohydrate moieties which are covalently attached to NTNR $\alpha$  when it is produced in the mammalian cell from which it is derived in nature. Accordingly, human NTNR $\alpha$  produced in a non-human cell is an example of a NTNR $\alpha$  which may "not be associated with native glycosylation." Sometimes, the NTNR $\alpha$  is unglycosylated (*e.g.*, as a result of being produced recombinantly in a prokaryote).

A "native sequence NTNR $\alpha$ " comprises a polypeptide having the same amino acid sequence as a NTNR $\alpha$  derived from nature. Thus, a native sequence NTNR $\alpha$  can have the amino acid sequence of naturally occurring rat NTNR $\alpha$ , murine NTNR $\alpha$ , human NTNR $\alpha$ , or NTNR $\alpha$  from any other mammalian species. Such native sequence NTNR $\alpha$  polypeptides can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence NTNR $\alpha$ " specifically encompasses naturally-occurring truncated forms of the NTNR $\alpha$ , naturally-occurring variant forms (*e.g.*, alternatively spliced forms), and naturally-occurring allelic variants of the NTNR $\alpha$ . The preferred native sequence NTNR $\alpha$  is a mature native sequence NTNR $\alpha$ . NTNR $\alpha$  sequence for human and rat are shown in Figures 1A-1B and 2A-2B. Preferred molecules are those comprising a nucleic acid molecule that is capable of hybridizing under moderate, and more preferably under stringent hybridization conditions, with the DNA sequence encoding the human NTN receptor shown in Figure 1A-1B. In one embodiment the NTNR $\alpha$  nucleic acid hybridizes at 42°C in 20% formamide with the DNA sequence encoding the NTN receptor shown in Figure 1A-1B. In another embodiment a nucleic acid molecule is capable of hybridizing at 42°C in 20% formamide with a DNA sequence of at least 10 contiguous bases, and preferably at least 20 contiguous bases, more preferably with at least 45 bases, and even more preferably with at least 60 bases encoding a portion of the complete NTN receptor shown in Figures 1A-1B or 2A-2B. Preferred sequences do not hybridize GDNFR $\alpha$  sequences under similar conditions.

The "NTNR $\alpha$  extracellular domain" (ECD) is a form of the NTNR $\alpha$  which is essentially free of the transmembrane and cytoplasmic domains of NTNR $\alpha$ , *i.e.*, has less than 1% of such domains, preferably 0.5 to 0% of such domains, and more preferably 0.1 to 0% of such domains. Ordinarily, the NTNR $\alpha$  ECD will have an amino acid sequence having at least about 60% amino acid sequence identity with the amino acid sequence of the ECD of an NTNR $\alpha$ , for example as indicated in Figures 1A-1B or 2A-2B for NTNR $\alpha$  or the corresponding sequences provided herein, *e.g.* mouse sequences, preferably at least about 65%, more preferably at least about 75%, even more preferably at least about 80%, even more preferably at least about 90%, with increasing preference of 95%, to at least 99% amino acid sequence identity, and finally to 100% identity, and thus includes NTNR $\alpha$  variants as defined below. Preferred sequences will be at least 16 amino acids long, preferably at least 20 amino acids long, and even more preferably at least 40 amino acids long.

"NTNR $\alpha$  variant" means a biologically active NTNR $\alpha$  as defined below having less than 100% sequence identity (but at least 60% identity) with a NTNR $\alpha$ , for example, having the deduced amino acid



sequence shown in Figures 1A-1B or 2A-2B for NTNR $\alpha$  or with the sequences provided herein. Such NTNR $\alpha$  variants include NTNR $\alpha$  polypeptides wherein one or more amino acid residues are added at the N- or C-terminus of, or within, a NTNR $\alpha$  sequence; from about one to thirty amino acid residues are deleted, and optionally substituted by one or more amino acid residues; and derivatives of the above polypeptides, wherein  
5 an amino acid residue has been covalently modified so that the resulting product has a non-naturally occurring amino acid. Ordinarily, a biologically active NTNR $\alpha$  variant will have an amino acid sequence having about 60% amino acid sequence identity with the amino acid sequence of a naturally-occurring NTNR $\alpha$  (e.g., as shown in Figures 1A-1B or 2A-2B or the corresponding sequences provided herein), preferably at least about 65%, more preferably at least about 75%, even more preferably at least about 80%, even more preferably at  
10 least about 90%, with increasing preference of 95%, to at least 99% amino acid sequence identity, and finally to 100% identity.

A "chimeric NTNR $\alpha$ " is a polypeptide comprising full-length NTNR $\alpha$  or one or more domains thereof (e.g., the extracellular domain) fused or bonded to heterologous polypeptide. The chimeric NTNR $\alpha$  will generally share at least one biological property in common with NTNR $\alpha$ . Examples of chimeric NTNR $\alpha$ s  
15 include immunoadhesins and epitope-tagged NTNR $\alpha$ .

The term "immunoadhesin" is used interchangeably with the expression "NTNR $\alpha$ -immunoglobulin chimera" and refers to a chimeric molecule that combines a portion of the NTNR $\alpha$  (generally the extracellular domain thereof) with an immunoglobulin sequence. The immunoglobulin sequence preferably, but not necessarily, is an immunoglobulin constant domain. The immunoglobulin moiety in the chimeras of the  
20 present invention may be obtained from IgG1, IgG2, IgG3 or IgG4 subtypes, IgA, IgE, IgD or IgM, but preferably IgG1 or IgG3.

The term "epitope-tagged" when used herein refers to a chimeric polypeptide comprising NTNR $\alpha$  fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody thereagainst can be made, yet is short enough such that it does not interfere with biological activity  
25 of the NTNR $\alpha$ . The tag polypeptide preferably also is fairly unique so that the antibody thereagainst does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8-50 amino acid residues (preferably between about 9-30 residues). Preferred are poly-histidine sequences, which bind nickel, allowing isolation of the tagged protein by Ni-NTA chromatography as described (Lindsay *et al. Neuron* 17:571-574 (1996)), for example.

30 "Isolated NTNR $\alpha$ " means NTNR $\alpha$  that has been purified from a NTNR $\alpha$  source or has been prepared by recombinant or synthetic methods and is sufficiently free of other peptides or proteins (1) to obtain at least 15 and preferably 20 amino acid residues of the N-terminal or of an internal amino acid sequence by using a spinning cup sequenator or the best commercially available amino acid sequenator marketed or as modified by published methods as of the filing date of this application, or (2) to homogeneity by SDS-PAGE under non-  
35 reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Homogeneity here means less than about 5% contamination with other source proteins.

"Essentially pure" protein means a composition comprising at least about 90% by weight of the protein, based on total weight of the composition, preferably at least about 95% by weight. "Essentially

homogeneous" protein means a composition comprising at least about 99% by weight of protein, based on total weight of the composition.

"Biological property" when used in conjunction with either "NTNR $\alpha$ " or "isolated NTNR $\alpha$ " means having an effector or antigenic function or activity that is directly or indirectly caused or performed by native sequence NTNR $\alpha$  (whether in its native or denatured conformation). Effector functions include ligand binding, and enhancement of survival, differentiation and/or proliferation of cells (especially proliferation of cells). However, effector functions do not include possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against native sequence NTNR $\alpha$ .

An "antigenic function" means possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against native sequence NTNR $\alpha$ . The principal antigenic function of a NTNR $\alpha$  polypeptide is that it binds with an affinity of at least about  $10^6$  L/mole to an antibody raised against native sequence NTNR $\alpha$ . Ordinarily, the polypeptide binds with an affinity of at least about  $10^7$  L/mole. The antibodies used to define "antigenic function" are rabbit polyclonal antibodies raised by formulating the NTNR $\alpha$  in Freund's complete adjuvant, subcutaneously injecting the formulation, and boosting the immune response by intraperitoneal injection of the formulation until the titer of the anti-NTNR $\alpha$  antibody plateaus.

"Biologically active" when used in conjunction with either "NTNR $\alpha$ " or "isolated NTNR $\alpha$ " means a NTNR $\alpha$  polypeptide that exhibits or shares an effector function of native sequence NTNR $\alpha$  and that may (but need not), in addition, possess an antigenic function. A principal effector function of the NTNR $\alpha$  is its ability to bind NTN. Another principal effector function of NTNR $\alpha$  is activating Ret tyrosine kinase (resulting in Ret autophosphorylation) to activate downstream pathways mediated by Ret signaling function.

"Antigenically active" NTNR $\alpha$  is defined as a polypeptide that possesses an antigenic function of NTNR $\alpha$  and that may (but need not) in addition possess an effector function.

"Percent amino acid sequence identity" with respect to the NTNR $\alpha$  sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues in the NTNR $\alpha$  sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the candidate NTNR $\alpha$  sequence shall be construed as affecting sequence identity or homology.

"NTN ligand" is a molecule which binds to and preferably activates native sequence NTNR $\alpha$ . The ability of a molecule to bind to NTNR $\alpha$  can be determined, for example, by the ability of the putative ligand to bind to NTNR $\alpha$  immunoadhesin coated on an assay plate, for example. Specificity of binding can be determined by comparing binding to GDNFR $\alpha$ . Competitive binding of NTN to NTNR $\alpha$  is a preferred property of the ligand. The thymidine incorporation assay provides another means for screening for ligands which activate NTNR $\alpha$  function.

A "thymidine incorporation assay" can be used to screen for molecules which activate the NTNR $\alpha$ . In order to perform this assay, IL-3 dependent Baf3 cells (Palacios *et al.*, *Cell*, 41:727-734 (1985)) are stably transfected with full length native sequence NTNR $\alpha$  as described herein and Ret. The NTNR $\alpha$ /Ret/Baf3 cells so generated are starved of IL-3 for 24 hours in a humidified incubator at 37°C in 5%CO<sub>2</sub> and air. Following IL-3 starvation, the cells are plated out in 96 well culture dishes with, or without, a test sample containing a

potential agonist (such test samples are optionally diluted) and cultured for 24 hours in a cell culture incubator. 20 $\mu$ l of serum free RPMI media containing 1 $\mu$ Ci of  $^3$ H thymidine is added to each well for the last 6-8 hours. The cells are then harvested in 96 well filter plates and washed with water. The filters are then counted using a Packard Top Count Microplate Scintillation Counter, for example. Agonists are expected to induce a statistically significant increase (to a P value of 0.05) in  $^3$ H uptake, relative to control. Preferred agonists leads to an increase in  $^3$ H uptake which is at least two fold of that of the control. Other assays are described herein.

An "isolated" NTNR $\alpha$  nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the NTNR $\alpha$  nucleic acid. An isolated NTNR $\alpha$  nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated NTNR $\alpha$  nucleic acid molecules therefore are distinguished from the NTNR $\alpha$  nucleic acid molecule as it exists in natural cells. However, an isolated NTNR $\alpha$  nucleic acid molecule includes NTNR $\alpha$  nucleic acid molecules contained in cells that ordinarily express NTNR $\alpha$  where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The expression "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies, antibody compositions with polyepitopic specificity, bispecific antibodies, diabodies, and single-chain molecules, as well as antibody fragments (e.g., Fab, F(ab')<sub>2</sub>, and Fv), so long as they exhibit the desired biological activity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical

except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al.*, *Nature*, 256: 495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567 (Cabilly *et al.*)). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al.*, 624-628 (1991) and Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (Cabilly *et al.*, *supra*; Morrison *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)).

"Humanized" forms of non-human (*e.g.*, murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al.*, *Nature*, 321:522-525 (1986); Reichmann *et al.*, *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992). The humanized antibody includes a Primatized<sup>TM</sup> antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

"Non-immunogenic in a human" means that upon contacting the polypeptide of interest in a physiologically acceptable carrier and in a therapeutically effective amount with the appropriate tissue of a human, no state of sensitivity or resistance to the polypeptide of interest is demonstrable upon the second administration of the polypeptide of interest after an appropriate latent period (e.g., 8 to 14 days).

5 By "agonist antibody" is meant an antibody which is a NTNR $\alpha$  ligand, able to activate native sequence NTNR $\alpha$ .

A "neutralizing antibody" is one which is able to block or significantly reduce an effector function of native sequence NTNR $\alpha$ . For example, a neutralizing antibody may inhibit or reduce NTNR $\alpha$  activation by a NTN ligand, as determined, for example, in a neurite survival assays, a NTN binding assay, or other  
10 assays taught herein or known in the art.

The phrase "enhancing proliferation of a cell" encompasses the step of increasing the extent of growth and/or reproduction of the cell relative to an untreated cell either *in vitro* or *in vivo*. An increase in cell proliferation in cell culture can be detected by counting the number of cells before and after exposure to a molecule of interest. The extent of proliferation can be quantified via microscopic examination of the degree  
15 of confluence. Cell proliferation can also be quantified using the thymidine incorporation assay described herein.

By "enhancing differentiation of a cell" is meant the act of increasing the extent of the acquisition or possession of one or more characteristics or functions which differ from that of the original cell (*i.e.* cell specialization). This can be detected by screening for a change in the phenotype of the cell (*e.g.*, identifying  
20 morphological changes in the cell).

"Physiologically acceptable" carriers, excipients, or stabilizers are ones which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular  
25 weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronic or polyethylene glycol  
30 (PEG).

As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.*, IgG1, IgG2, IgG3, and IgG4) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule. Exemplary salvage receptor binding epitope sequences include HQNLSDGK; HQNISDGK; HQSLGTQ; VISSLGQ; and PKNSSMISNTP.

35 The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH),

thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor- $\alpha$  and - $\beta$ ; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); neurotrophic factors or nerve growth factors such as NGF- $\beta$ , NT-3, NT-4, NT-6, BDNF, CNTF, GDNF, AL-1 and other eph-receptor family ligands; platelet-growth factor; transforming growth factors (TGFs) such as TGF- $\alpha$  and TGF- $\beta$ ; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon- $\alpha$ , - $\beta$ , and - $\gamma$ ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1 $\alpha$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines. Also included are genetically engineered molecules with cytokine activity such as TrkA-IgG or other soluble receptor chimeras.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

By "solid phase" is meant a non-aqueous matrix to which a reagent of interest (e.g., the NTNR $\alpha$  or an antibody thereto) can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g., an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Patent No. 4,275,149.

Modes for carrying out the invention are presented herein. Glial cell line-derived neurotrophic factor ("GDNF") and Neurturin ("NTN") are two structurally related, potent survival factors for sympathetic sensory and central nervous system neurons (Lin *et al. Science* 260:1130-1132 (1993); Henderson *et al. Science* 266:1062-1064 (1994); Buj-Bello *et al., Neuron* 15:821-828 (1995); Kotzbauer *et al. Nature* 384:467-470 (1996)). Whereas GDNF was shown to mediate its actions through a multi-component receptor system composed of a ligand binding glycosyl-phosphatidyl inositol (GPI) linked protein (designated GDNFR $\alpha$ ) and the transmembrane tyrosine kinase Ret (Treanor *et al. Nature* 382:80-83 (1996); Jing *et al. Cell* 85:1113-1124 (1996); Trupp *et al. Nature* 381:785-789 (1996); Durbec *et al. Nature* 381:789-793 (1996)), the mechanism by which the NTN signal is transmitted has not been previously elucidated. Described herein is the isolation, sequence, and tissue distribution of a GPI-linked protein and its gene, designated NTNR $\alpha$ , which is shown to modulate response to NTN but not GDNF. It is shown herein that it is structurally related to GDNFR $\alpha$ . Using recombinant proteins in a cell free system, it is shown that NTNR $\alpha$  binds NTN (K<sub>d</sub> ~10pM) but not GDNF, and that NTN does not bind GDNFR $\alpha$  with a high affinity. Also shown is that cellular responses to NTN

require the presence of NTN $\alpha$ . Ligand bound NTN $\alpha$  induces phosphorylation of the tyrosine kinase receptor Ret. These findings identify Ret and NTN $\alpha$ , respectively, as signaling and ligand binding components of a receptor for NTN and related ligands. This defines a novel neurotrophic and differentiation factor receptor family of receptors containing a shared transmembrane protein tyrosine kinase (Ret) and a  
 5 ligand specific GPI-linked protein (NTN $\alpha$ ).

Glial cell line-derived neurotrophic factor ("GDNF") (Lin *et al.*, *Science*, 260:1130-1132 (1993); WO 93/06116, which are incorporated herein in its entirety), is a potent survival factor for midbrain dopaminergic (Lin *et al.*, *Science*, 260:1130-1132 (1993); Strömberg *et al.*, *Exp. Neurol.*, 124:401-412 (1993); Beck *et al.*, *Nature*, 373:339-341 (1995); Kearns *et al.*, *Brain Res.*, 672:104-111 (1995); Tomac *et al.*, *Nature*, 373:335-  
 10 339 (1995)) spinal motor (Henderson *et al.*, *Science*, 266:1062-1064 (1994); Oppenheim *et al.*, *Nature*, 373:344-346 (1995); Yan *et al.*, *Nature*, 373:341-344 (1995)) and noradrenergic neurons (Arenas *et al.*, *Neuron*, 15:1465-1473 (1995)), which degenerate in Parkinson's disease (Hirsch *et al.*, *Nature*, 334:345-348 (1988); Hornykiewicz *Mt. Sinai J. Med.*, 55:11-20 (1988)), amyotrophic lateral sclerosis (Hirano, *Amyotrophic Lateral Sclerosis and Other Motor Neuron Diseases*, P. Rowland, ed. (New York: Raven Press, Inc.) pp. 91-101 (1991)), and Alzheimer's disease (Marcyniuk *et al.*, *J. Neurol. Sci.*, 76:335-345 (1986); Cash  
 15 *et al.*, *Neurology*, 37:42-46 (1987); Chan-Palay *et al.*, *Comp. Neurol.*, 287:373-392 (1989)) respectively. Based on mice genetically engineered to lack GDNF, additional biological roles for GDNF have been reported: the development and/or survival of enteric, sympathetic, and sensory neurons and the renal system, but not for catecholaminergic neurons in the central nervous system (CNS) (Moore *et al.* *Nature* 382:76-79 (1996);  
 20 Pichel *et al.* *Nature* 382:73-76 (1996); Sanchez *et al.* *Nature* 382:70-73 (1996)). Despite the physiological and clinical importance of GDNF, little is known about its mechanism of action.

Cytokine receptors frequently assemble into multi-subunit complexes. Sometimes, the  $\alpha$  subunit of this complex is involved in binding the cognate growth factor and the  $\beta$ -subunit may contain an ability to transduce a signal to the cell. Without wishing to be bound by theory, these receptors have been assigned to  
 25 three subfamilies depending on the complexes formed. Subfamily 1 includes the receptors for EPO, granulocyte colony-stimulating factor (G-CSF), interleukin-4 (IL-4), interleukin-7 (IL-7), growth hormone (GH), and prolactin (PRL). Ligand binding to receptors belonging to this subfamily is thought to result in homodimerization of the receptor. Subfamily 2 includes receptors for IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-5 (IL-5), interleukin-6 (IL-6), leukemia inhibitory factor (LIF),  
 30 oncostatin M (OSM), and ciliary neurotrophic factor (CNTF). Subfamily 2 receptors are heterodimers having an  $\alpha$ -subunit for ligand binding, and  $\beta$ -subunit (either the shared  $\beta$ -subunit of the IL-3, GM-CSF, and IL-5 receptors or the gp130 subunit of the IL-6, LIF, OSM, and CNTF receptors) for signal transduction. Subfamily 3 contains only the interleukin-2 (IL-2) receptor. The  $\beta$  and  $\gamma$  subunits of the IL-2 receptor complex are cytokine-receptor polypeptides which associate with the  $\alpha$ -subunit of the unrelated Tac antigen.

35 The present invention is based on the discovery of the NTN $\alpha$ , a protein that binds NTN with a high affinity. The experiments described herein demonstrate that this molecule is a receptor which appears to play a role in mediating responses to NTN. In particular, this receptor has been found to be present in a variety of tissue and cell populations, including neurons, thus indicating that NTN ligands, such as agonist antibodies,

can be used to stimulate proliferation, growth, survival, differentiation, metabolism, or regeneration of NTNR $\alpha$ - and Ret-containing cells.

Techniques suitable for the production of NTNR $\alpha$  are well known in the art and include isolating NTNR $\alpha$  from an endogenous source of the polypeptide, peptide synthesis (using a peptide synthesizer) and  
5 recombinant techniques (or any combination of these techniques). The preferred technique for production of NTNR $\alpha$  is a recombinant technique to be described below.

Most of the discussion below pertains to recombinant production of NTNR $\alpha$  by culturing cells transformed with a vector containing NTNR $\alpha$  nucleic acid and recovering the polypeptide from the cell culture. It is further envisioned that the NTNR $\alpha$  of this invention may be produced by homologous  
10 recombination, as provided for in WO 91/06667, published 16 May 1991.

Briefly, this method involves transforming primary human cells containing a NTNR $\alpha$ -encoding gene with a construct (*i.e.*, vector) comprising an amplifiable gene (such as dihydrofolate reductase (DHFR) or others discussed below) and at least one flanking region of a length of at least about 150 bp that is homologous with a DNA sequence at the locus of the coding region of the NTNR $\alpha$  gene to provide amplification of the  
15 NTNR $\alpha$  gene. The amplifiable gene must be at a site that does not interfere with expression of the NTNR $\alpha$  gene. The transformation is conducted such that the construct becomes homologously integrated into the genome of the primary cells to define an amplifiable region.

Primary cells comprising the construct are then selected for by means of the amplifiable gene or other marker present in the construct. The presence of the marker gene establishes the presence and integration of  
20 the construct into the host genome. No further selection of the primary cells need be made, since selection will be made in the second host. If desired, the occurrence of the homologous recombination event can be determined by employing PCR and either sequencing the resulting amplified DNA sequences or determining the appropriate length of the PCR fragment when DNA from correct homologous integrants is present and expanding only those cells containing such fragments. Also if desired, the selected cells may be amplified at  
25 this point by stressing the cells with the appropriate amplifying agent (such as methotrexate if the amplifiable gene is DHFR), so that multiple copies of the target gene are obtained. Preferably, however, the amplification step is not conducted until after the second transformation described below.

After the selection step, DNA portions of the genome, sufficiently large to include the entire amplifiable region, are isolated from the selected primary cells. Secondary mammalian expression host cells  
30 are then transformed with these genomic DNA portions and cloned, and clones are selected that contain the amplifiable region. The amplifiable region is then amplified by means of an amplifying agent if not already amplified in the primary cells. Finally, the secondary expression host cells now comprising multiple copies of the amplifiable region containing NTNR $\alpha$  are grown so as to express the gene and produce the protein.

The conserved structure and sequence of the mammalian NTNR $\alpha$  and the elucidation of the cDNA  
35 sequence which encodes the rat and mouse receptor, as well as human sequences disclosed herein, make it possible to clone gene sequences from other mammals which encode the NTNR $\alpha$ . Of particular interest to the present invention is the ability to clone the human NTNR $\alpha$  molecules using the sequences disclosed herein. The DNA encoding NTNR $\alpha$  may be obtained from any cDNA library prepared from tissue believed to possess the NTNR $\alpha$  mRNA and to express it at a detectable level, as shown herein in the Examples. Accordingly,



NTNR $\alpha$  DNA can be conveniently obtained from a cDNA library prepared, for example, from mammalian fetal liver, brain, muscle, intestine, and peripheral nerves. The NTNR $\alpha$ -encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries are screened with probes (such as antibodies to the NTNR $\alpha$  or oligonucleotides of about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures as described in chapters 10-12 of Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding NTNR $\alpha$  is to use PCR methodology as described in section 14 of Sambrook *et al.*, *supra*.

A preferred method of practicing this invention is to use carefully selected oligonucleotide sequences to screen cDNA libraries from various human tissues, preferably human fetal liver. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. Preferred sequences are obtained from the naturally-occurring NTNR $\alpha$  disclosed herein.

The oligonucleotide must be labeled such that it can be detected upon hybridization to DNA in the library being screened. The preferred method of labeling is to use <sup>32</sup>P-labeled ATP with polynucleotide kinase, as is well known in the art, to radiolabel the oligonucleotide. However, other methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labeling.

Amino acid sequence variants of NTNR $\alpha$  are prepared by introducing appropriate nucleotide changes into the NTNR $\alpha$  DNA, or by synthesis of the desired NTNR $\alpha$  polypeptide. Such variants represent insertions, substitutions, and/or specified deletions of, residues within or at one or both of the ends of the amino acid sequence of a naturally occurring NTNR $\alpha$ , such as the NTNR $\alpha$  shown in Figures 1A-1B or 2A-2B or sequences disclosed herein. Preferably, these variants represent insertions and/or substitutions within or at one or both ends of the mature sequence, and/or insertions, substitutions and/or specified deletions within or at one or both of the ends of the signal sequence of the NTNR $\alpha$ . Any combination of insertion, substitution, and/or specified deletion is made to arrive at the final construct, provided that the final construct possesses the desired biological activity as defined herein. The amino acid changes also may alter post-translational processes of the NTNR $\alpha$ , such as changing the number or position of glycosylation sites, altering the membrane anchoring characteristics, and/or altering the intracellular location of the NTNR $\alpha$  by inserting, deleting, or otherwise affecting the leader sequence of the NTNR $\alpha$ .

Variations in the native sequence as described above can be made using any of the techniques and guidelines for conservative and non-conservative mutations set forth in U.S. Pat. No. 5,364,934. These include oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. See also, for example, Table I therein and the discussion surrounding this table for guidance on selecting amino acids to change, add, or delete.

The nucleic acid (*e.g.*, cDNA or genomic DNA) encoding the NTNR $\alpha$  is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

The NTNR $\alpha$ s of this invention may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the NTNR $\alpha$  DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native NTNR $\alpha$  signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, *e.g.*, the yeast invertase leader,  $\alpha$  factor leader (including *Saccharomyces* and *Kluyveromyces*  $\alpha$ -factor leaders, the latter described in U.S. Pat. No. 5,010,182 issued 23 April 1991), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native signal sequence (*e.g.*, the NTNR $\alpha$  presequence that normally directs secretion of NTNR $\alpha$  from human cells *in vivo*) is satisfactory, although other mammalian signal sequences may be suitable, such as signal sequences from other animal NTNR $\alpha$ s, and signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex gD signal.

The DNA for such precursor region is ligated in reading frame to DNA encoding the mature NTNR $\alpha$  or a soluble variant thereof.

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 $\mu$  plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, *i.e.*, they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of NTNR $\alpha$  DNA. However, the recovery of genomic DNA encoding NTNR $\alpha$  is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the NTNR $\alpha$  DNA.

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic acid and hygromycin.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the NTNR $\alpha$  nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes NTNR $\alpha$ . Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of NTNR $\alpha$  are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, *etc.* A preferred vector system is provided in U.S. Patent No. 5,561,053.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub *et al.*, *Proc. Natl. Acad. Sci. USA*, 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding NTNR $\alpha$ . This amplification technique can be used with any otherwise suitable host, *e.g.*, ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding NTNR $\alpha$ , wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, *e.g.*, kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 (Stinchcomb *et al.*, *Nature*, 282:39 (1979)). The *trp1* gene provides a selection marker for a mutant strain of

yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. Jones, *Genetics*, 85:12 (1977). The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

- 5 In addition, vectors derived from the 1.6  $\mu$ m circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts. Bianchi *et al.*, *Curr. Genet.*, 12:185 (1987). More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis*. Van den Berg, *Bio/Technology*, 8:135 (1990). Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed. Fleer *et al.*,  
10 *Bio/Technology*, 9:968-975 (1991).

- Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the NTNR $\alpha$  nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the NTNR $\alpha$  nucleic acid sequence, to which they  
15 are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, *e.g.*, the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to NTNR $\alpha$ -encoding DNA by removing the promoter from the  
20 source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native NTNR $\alpha$  promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the NTNR $\alpha$  DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of NTNR $\alpha$  as compared to the native NTNR $\alpha$  promoter.

- 25 Promoters suitable for use with prokaryotic hosts include the  $\beta$ -lactamase and lactose promoter systems (Chang *et al.*, *Nature*, 275:615 (1978); Goeddel *et al.*, *Nature*, 281:544 (1979)), alkaline phosphatase, a tryptophan (*trp*) promoter system (Goeddel, *Nucleic Acids Res.*, 8:4057 (1980); EP 36,776), and hybrid promoters such as the *tac* promoter. deBoer *et al.*, *Proc. Natl. Acad. Sci. USA*, 80:21-25 (1983). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby  
30 enabling a skilled worker operably to ligate them to DNA encoding NTNR $\alpha$  (Siebenlist *et al.*, *Cell*, 20:269 (1980)) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Delgarno (S.D.) sequence operably linked to the DNA encoding NTNR $\alpha$ .

- Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence  
35 found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al.*, *J. Biol. Chem.*, 255:2073 (1980)) or other glycolytic enzymes (Hess *et al.*, *J. Adv. Enzyme Reg.*, 7:149 (1968); Holland, *Biochemistry*, 17:4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

NTNR $\alpha$  transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the NTNR $\alpha$  sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. Fiers *et al.*, *Nature*, 273:113 (1978); Mulligan *et al.*, *Science*, 209:1422-1427 (1980); Pavlakis *et al.*, *Proc. Natl. Acad. Sci. USA*, 78:7398-7402 (1981). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a *Hind*III E restriction fragment. Greenaway *et al.*, *Gene*, 18:355-360 (1982). A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. See also Gray *et al.*, *Nature*, 295:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes *et al.*, *Nature*, 297:598-601 (1982) on expression of human  $\beta$ -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani *et al.*, *Proc. Natl. Acad. Sci. USA*, 79:5166-5170 (1982) on expression of the human interferon  $\beta$ 1 gene in cultured mouse and rabbit cells; and Gorman *et al.*, *Proc. Natl. Acad. Sci. USA*, 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

Transcription of a DNA encoding the NTNR $\alpha$  of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' (Laimins *et al.*, *Proc. Natl. Acad. Sci. USA*, 78:993 (1981)) and 3' (Lusky *et al.*, *Mol. Cell Bio.*, 3:1108 (1983)) to the transcription unit, within an intron (Banerji *et al.*, *Cell*, 33:729 (1983)), as well as within the coding sequence itself. Osborne *et al.*, *Mol. Cell*

*Bio.*, 4:1293 (1984). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin,  $\alpha$ -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature*, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the NTNR $\alpha$ -encoding sequence, but is preferably located at a site 5' from the promoter.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding NTNR $\alpha$ .

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing *et al.*, *Nucleic Acids Res.*, 9:309 (1981) or by the method of Maxam *et al.*, *Methods in Enzymology*, 65:499 (1980).

Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding NTNR $\alpha$ . In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Sambrook *et al.*, *supra*, pp. 16.17 - 16.22. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of NTNR $\alpha$  that are biologically active NTNR $\alpha$ .

Other methods, vectors, and host cells suitable for adaptation to the synthesis of NTNR $\alpha$  in recombinant vertebrate cell culture are described in Gething *et al.*, *Nature*, 293:620-625 (1981); Mantei *et al.*, *Nature*, 281:40-46 (1979); EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of NTNR $\alpha$  is pRK5 (EP 307,247) or pSV16B. WO 91/08291 published 13 June 1991.

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*,

and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting. Strain W3110 is a particularly preferred host or parent host because it is a common host strain for recombinant DNA product fermentations. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes. For example, strain W3110 may be modified to effect a genetic mutation in the genes encoding proteins, with examples of such hosts including *E. coli* W3110 strain 27C7. The complete genotype of 27C7 is *tonAΔ ptr3 phoAΔE15 Δ(argF-lac)169 ompTΔ degP41 karF*. Strain 27C7 was deposited on 30 October 1991 in the American Type Culture Collection as ATCC No. 55,244. Alternatively, the strain of *E. coli* having mutant periplasmic protease disclosed in U.S. Patent No. 4,946,783 issued 7 August 1990 may be employed. Alternatively still, methods of cloning, e.g., PCR or other nucleic acid polymerase reactions, are suitable.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for NTNR $\alpha$ -encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe* (Beach *et al.*, *Nature*, 290:140 (1981); EP 139,383 published 2 May 1985); *Kluyveromyces* hosts (U.S. Patent No. 4,943,529; Fleer *et al.*, *supra*) such as, e.g., *K. lactis* (MW98-8C, CBS683, CBS4574; Louvencourt *et al.*, *J. Bacteriol.*, 737 (1983)), *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickerhamii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilae* (ATCC 36,906; Van den Berg *et al.*, *supra*), *K. thermotolerans*, and *K. marxianus*; *yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070; Sreekrishna *et al.*, *J. Basic Microbiol.*, 28:265-278 (1988)); *Candida*; *Trichoderma reesia* (EP 244,234); *Neurospora crassa* (Case *et al.*, *Proc. Natl. Acad. Sci. USA*, 76:5259-5263 (1979)); *Schwanniomyces* such as *Schwanniomyces occidentalis* (EP 394,538 published 31 October 1990); and filamentous fungi such as, e.g., *Neurospora*, *Penicillium*, *Tolypocladium* (WO 91/00357 published 10 January 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance *et al.*, *Biochem. Biophys. Res. Commun.*, 112:284-289 (1983); Tilburn *et al.*, *Gene*, 26:205-221 (1983); Yelton *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:1470-1474 (1984)) and *A. niger*. Kelly *et al.*, *EMBO J.*, 4:475-479 (1985).

Suitable host cells for the expression of glycosylated NTNR $\alpha$  are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. See, e.g., Luckow *et al.*, *Bio/Technology*, 6:47-55 (1988); Miller *et al.*, in *Genetic Engineering*, Setlow *et al.*, eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda *et al.*, *Nature*, 315:592-594 (1985). A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*, which has been previously manipulated to contain the NTNR $\alpha$ -encoding DNA. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the NTNR $\alpha$  is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the NTNR $\alpha$ -encoding DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker *et al.*, *J. Mol. Appl. Gen.*, 1:561 (1982). In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. EP 321,196 published 21 June 1989.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. See, *e.g.*, *Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973). Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, *J. Gen Virol.*, 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub *et al.*, *Proc. Natl. Acad. Sci. USA*, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.*, 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, *Annals N.Y. Acad. Sci.*, 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for NTNR $\alpha$  production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, CaPO<sub>4</sub> and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook *et al.*, *supra*, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw *et al.*, *Gene*, 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham *et al.*, *Virology*, 52:456-457 (1978) is preferred. General aspects of mammalian cell host system



transformations have been described in U.S. Pat. No. 4,399,216 issued 16 August 1983. Transformations into yeast are typically carried out according to the method of Van Solingen *et al.*, *J. Bact.*, 130:946 (1977) and Hsiao *et al.*, *Proc. Natl. Acad. Sci. USA*, 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, *e.g.*, polybrene, polyornithine, *etc.*, may also be used. For various techniques for transforming mammalian cells, see Keown *et al.*, *Methods in Enzymology*, 185:527-537 (1990) and Mansour *et al.*, *Nature*, 336:348-352 (1988).

Prokaryotic cells used to produce the NTN $\alpha$  polypeptide of this invention are cultured in suitable media as described generally in Sambrook *et al.*, *supra*.

The mammalian host cells used to produce the NTN $\alpha$  of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al. Meth. Enz.*, 58:44 (1979), Barnes *et al.*, *Anal. Biochem.*, 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN<sup>TM</sup> drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in *Mammalian Cell Biotechnology: a Practical Approach*, M. Butler, ed. (IRL Press, 1991).

The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (Thomas, *Proc. Natl. Acad. Sci. USA*, 77:5201-5205 (1980)), dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly <sup>32</sup>P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound

to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, can be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu *et al.*, *Am. J. Clin. Path.*, 75:734-738 (1980).

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared as described herein.

NTNR $\alpha$  (e.g., NTNR $\alpha$  ECD) preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates. If the NTNR $\alpha$  is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100).

When NTNR $\alpha$  is produced in a recombinant cell other than one of human origin, the NTNR $\alpha$  is completely free of proteins or polypeptides of human origin. However, it is necessary to purify NTNR $\alpha$  from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to NTNR $\alpha$ . As a first step, the culture medium or lysate can be centrifuged to remove particulate cell debris. NTNR $\alpha$  can then be purified from contaminant soluble proteins and polypeptides with the following procedures, which are exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica; chromatofocusing; immunoaffinity; epitope-tag binding resin; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

NTNR $\alpha$  variants in which residues have been deleted, inserted, or substituted are recovered in the same fashion as native sequence NTNR $\alpha$ , taking account of any substantial changes in properties occasioned by the variation. Immunoaffinity resins, such as a monoclonal anti-NTNR $\alpha$  resin, can be employed to absorb the NTNR $\alpha$  variant by binding it to at least one remaining epitope.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants.

Covalent modifications of NTNR $\alpha$  polypeptides are included within the scope of this invention. Both native sequence NTNR $\alpha$  and amino acid sequence variants of the NTNR $\alpha$  may be covalently modified. One type of covalent modification of the NTNR $\alpha$  is introduced into the molecule by reacting targeted amino acid residues of the NTNR $\alpha$  with an organic derivatizing agent that is capable of reacting the N-terminal residue, the C-terminal residue, or with selected side chains.

CysteinyI residues most commonly are reacted with  $\alpha$ -haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. CysteinyI residues also are derivatized by reaction with bromotrifluoroacetone,  $\alpha$ -bromo- $\beta$ -(5-imidazolyl)propionic acid,

chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction  
5 is preferably performed in 0.1M sodium cacodylate at pH 6.0.

Lysyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysyl residues. Other suitable reagents for derivatizing  $\alpha$ -amino-containing residues include imidoesters such as methyl picolinimide, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-  
10 pentanedione, and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed under alkaline conditions because of the high  $pK_a$  of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as with the arginine  
15 epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using  $^{125}I$  or  $^{131}I$  to prepare labeled proteins  
20 for use in radioimmunoassay, the chloramine T method being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides ( $R-N=C=N-R'$ ), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions. \*

Derivatization with bifunctional agents is useful for crosslinking NTNR $\alpha$  to a water-insoluble support matrix or surface for use in the method for purifying anti-NTNR $\alpha$  antibodies, and vice-versa. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-  
30 1,8-octane. Derivatizing agents such as methyl-3-((p-azidophenyl)dithio)propioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

35 Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. These residues are deamidated under neutral or basic conditions. The deamidated form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and histidine side chains

(T.E. Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the NTN $\alpha$  polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. By altering is meant deleting  
5 one or more carbohydrate moieties found in native NTN $\alpha$ , and/or adding one or more glycosylation sites that are not present in the native NTN $\alpha$ .

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the  
10 recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose to a hydroxylamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

15 Addition of glycosylation sites to the NTN $\alpha$  polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native NTN $\alpha$  sequence (for O-linked glycosylation sites). For ease, the NTN $\alpha$  amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating  
20 the DNA encoding the NTN $\alpha$  polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above and in U.S. Pat. No. 5,364,934, *supra*.

Another means of increasing the number of carbohydrate moieties on the NTN $\alpha$  polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. These procedures are advantageous in that  
25 they do not require production of the polypeptide in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in  
30 WO 87/05330 published 11 September 1987, and in Aplin *et al.*, *CRC Crit. Rev. Biochem.*, 259-306 (1981).

Removal of carbohydrate moieties present on the NTN $\alpha$  polypeptide may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the  
35 polypeptide intact. Chemical deglycosylation is described by Hakimuddin, *et al.*, *Arch. Biochem. Biophys.*, 259:52 (1987) and by Edge *et al.*, *Anal. Biochem.*, 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al.*, *Meth. Enzymol.*, 138:350 (1987).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin *et al.*, *J. Biol. Chem.*, 257:3105 (1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of NTNR $\alpha$  comprises linking the NTNR $\alpha$  polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

Variants can be assayed as taught herein. A change in the immunological character of the NTNR $\alpha$  molecule, such as affinity for a given antibody, can be measured by a competitive-type immunoassay. Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, or the tendency to aggregate with carriers or into multimers are assayed by methods well known in the art.

This invention encompasses chimeric polypeptides comprising NTNR $\alpha$  fused to a heterologous polypeptide. A chimeric NTNR $\alpha$  is one type of NTNR $\alpha$  variant as defined herein. In one preferred embodiment, the chimeric polypeptide comprises a fusion of the NTNR $\alpha$  with a tag polypeptide which provides an epitope to which an anti-tag antibody or molecule can selectively bind. The epitope-tag is generally provided at the amino- or carboxyl- terminus of the NTNR $\alpha$ . Such epitope-tagged forms of the NTNR $\alpha$  are desirable, as the presence thereof can be detected using a labeled antibody against the tag polypeptide. Also, provision of the epitope tag enables the NTNR $\alpha$  to be readily purified by affinity purification using the anti-tag antibody. Affinity purification techniques and diagnostic assays involving antibodies are described later herein.

Tag polypeptides and their respective antibodies are well known in the art. Examples include the flu HA tag polypeptide and its antibody 12CA5 (Field *et al.*, *Mol. Cell. Biol.*, 8:2159-2165 (1988)); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan *et al.*, *Molecular and Cellular Biology*, 5:3610-3616 (1985)); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody. Paborsky *et al.*, *Protein Engineering*, 3(6):547-553 (1990). Other tag polypeptides have been disclosed. Examples include the Flag-peptide (Hopp *et al.*, *BioTechnology*, 6:1204-1210 (1988)); the KT3 epitope peptide (Martin *et al.*, *Science*, 255:192-194 (1992)); an  $\alpha$ -tubulin epitope peptide (Skinner *et al.*, *J. Biol. Chem.*, 266:15163-15166 (1991)); and the T7 gene 10 protein peptide tag. Lutz-Freyermuth *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:6393-6397 (1990). Once the tag polypeptide has been selected, an antibody thereto can be generated using the techniques disclosed herein. A C-terminal poly-histidine sequence tag is preferred. Poly-histidine sequences allow isolation of the tagged protein by Ni-NTA chromatography as described (Lindsay *et al. Neuron* 17:571-574 (1996)), for example.

The general methods suitable for the construction and production of epitope-tagged NTNR $\alpha$  are the same as those disclosed hereinabove. NTNR $\alpha$ -tag polypeptide fusions are most conveniently constructed by fusing the cDNA sequence encoding the NTNR $\alpha$  portion in-frame to the tag polypeptide DNA sequence and expressing the resultant DNA fusion construct in appropriate host cells. Ordinarily, when preparing the NTNR $\alpha$ -tag polypeptide chimeras of the present invention, nucleic acid encoding the NTNR $\alpha$  will be fused

at its 3' end to nucleic acid encoding the N-terminus of the tag polypeptide, however 5' fusions are also possible.

Epitope-tagged NTNR $\alpha$  can be conveniently purified by affinity chromatography using the anti-tag antibody. The matrix to which the affinity antibody is attached is most often agarose, but other matrices are available (e.g. controlled pore glass or poly(styrenedivinyl)benzene). The epitope-tagged NTNR $\alpha$  can be eluted from the affinity column by varying the buffer pH or ionic strength or adding chaotropic agents, for example.

Chimeras constructed from a receptor sequence linked to an appropriate immunoglobulin constant domain sequence (immunoadhesins) are known in the art. Immunoadhesins reported in the literature include fusions of the T cell receptor\* (Gascoigne *et al.*, *Proc. Natl. Acad. Sci. USA*, 84: 2936-2940 (1987)); CD4\* (Capon *et al.*, *Nature* 337: 525-531 (1989); Traunecker *et al.*, *Nature*, 339: 68-70 (1989); Zettmeissl *et al.*, *DNA Cell Biol. USA*, 9: 347-353 (1990); Byrn *et al.*, *Nature*, 344: 667-670 (1990)); L-selectin (homing receptor) ((Watson *et al.*, *J. Cell. Biol.*, 110:2221-2229 (1990); Watson *et al.*, *Nature*, 349: 164-167 (1991)); CD44\* (Aruffo *et al.*, *Cell*, 61: 1303-1313 (1990)); CD28\* and B7\* (Linsley *et al.*, *J. Exp. Med.*, 173: 721-730 (1991)); CTLA-4\* (Lisley *et al.*, *J. Exp. Med.* 174: 561-569 (1991)); CD22\* (Stamenkovic *et al.*, *Cell*, 66:1133-1144 (1991)); TNF receptor (Ashkenazi *et al.*, *Proc. Natl. Acad. Sci. USA*, 88: 10535-10539 (1991); Lesslauer *et al.*, *Eur. J. Immunol.*, 27: 2883-2886 (1991); Peppel *et al.*, *J. Exp. Med.*, 174:1483-1489 (1991)); NP receptors (Bennett *et al.*, *J. Biol. Chem.* 266:23060-23067 (1991)); and IgE receptor  $\alpha$ \* (Ridgway *et al.*, *J. Cell. Biol.*, 115:abstr. 1448 (1991)), where the asterisk (\*) indicates that the receptor is member of the immunoglobulin superfamily.

The simplest and most straightforward immunoadhesin design combines the binding region(s) of the "adhesin" protein with the hinge and Fc regions of an immunoglobulin heavy chain. Ordinarily, when preparing the NTNR $\alpha$ -immunoglobulin chimeras of the present invention, nucleic acid encoding the extracellular domain of the NTNR $\alpha$  will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence, however N-terminal fusions are also possible.

Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge and CH2 and CH3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the CH1 of the heavy chain or the corresponding region of the light chain.

The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion or binding characteristics of the NTNR $\alpha$ -immunoglobulin chimeras.

In some embodiments, the NTNR $\alpha$ -immunoglobulin chimeras are assembled as monomers, or hetero- or homo-multimer, and particularly as dimers or tetramers, essentially as illustrated in WO 91/08298.

In a preferred embodiment, the NTNR $\alpha$  extracellular domain sequence is fused to the N-terminus of the C-terminal portion of an antibody (in particular the Fc domain), containing the effector functions of an immunoglobulin, e.g. immunoglobulin G<sub>1</sub> (IgG<sub>1</sub>). It is possible to fuse the entire heavy chain constant region to the NTNR $\alpha$  extracellular domain sequence. However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site (which defines IgG Fc chemically; residue 216, taking the first

residue of heavy chain constant region to be 114, or analogous sites of other immunoglobulins) is used in the fusion. In a particularly preferred embodiment, the NTN $\alpha$  amino acid sequence is fused to the hinge region and CH2 and CH3, or to the CH1, hinge, CH2 and CH3 domains of an IgG1, IgG2, or IgG3 heavy chain. The precise site at which the fusion is made is not critical, and the optimal site can be determined by routine experimentation.

In some embodiments, the NTN $\alpha$ -immunoglobulin chimeras are assembled as multimer, and particularly as homo-dimers or -tetramers. Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each four unit may be the same or different.

Alternatively, the NTN $\alpha$  extracellular domain sequence can be inserted between immunoglobulin heavy chain and light chain sequences such that an immunoglobulin comprising a chimeric heavy chain is obtained. In this embodiment, the NTN $\alpha$  sequence is fused to the 3' end of an immunoglobulin heavy chain in each arm of an immunoglobulin, either between the hinge and the CH2 domain, or between the CH2 and CH3 domains. Similar constructs have been reported by Hoogenboom *et al.*, *Mol. Immunol.*, 28:1027-1037 (1991).

Although the presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention, an immunoglobulin light chain might be present either covalently associated to an NTN $\alpha$ -immunoglobulin heavy chain fusion polypeptide, or directly fused to the NTN $\alpha$  extracellular domain. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the NTN $\alpha$ -immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs. Methods suitable for the preparation of such structures are, for example, disclosed in U.S. Patent No. 4,816,567 issued 28 March 1989.

In a preferred embodiment, the immunoglobulin sequences used in the construction of the immunoadhesins of the present invention are from an IgG immunoglobulin heavy chain constant domain. For human immunoadhesins, the use of human IgG1 and IgG3 immunoglobulin sequences is preferred. A major advantage of using IgG1 is that IgG1 immunoadhesins can be purified efficiently on immobilized protein A. In contrast, purification of IgG3 requires protein G, a significantly less versatile medium. However, other structural and functional properties of immunoglobulins should be considered when choosing the Ig fusion partner for a particular immunoadhesin construction. For example, the IgG3 hinge is longer and more flexible, so it can accommodate larger adhesin domains that may not fold or function properly when fused to IgG1. Another consideration may be valency; IgG immunoadhesins are bivalent homodimers, whereas Ig subtypes like IgA and IgM may give rise to dimeric or pentameric structures, respectively, of the basic Ig homodimer unit. For NTN $\alpha$  immunoadhesins designed for *in vivo* application, the pharmacokinetic properties and the effector functions specified by the Fc region are important as well. Although IgG1, IgG2 and IgG4 all have *in vivo* half-lives of 21 days, their relative potencies at activating the complement system are different. IgG4 does not activate complement, and IgG2 is significantly weaker at complement activation than IgG1.

Moreover, unlike IgG1, IgG2 does not bind to Fc receptors on mononuclear cells or neutrophils. While IgG3 is optimal for complement activation, its *in vivo* half-life is approximately one third of the other IgG isotypes. Another important consideration for immunoadhesins designed to be used as human therapeutics is the number of allotypic variants of the particular isotype. In general, IgG isotypes with fewer serologically-defined allotypes are preferred. For example, IgG1 has only four serologically-defined allotypic sites, two of which (G1m and 2) are located in the Fc region; and one of these sites G1m1, is non-immunogenic. In contrast, there are 12 serologically-defined allotypes in IgG3, all of which are in the Fc region; only three of these sites (G3m5, 11 and 21) have one allotype which is nonimmunogenic. Thus, the potential immunogenicity of a  $\gamma$ 3 immunoadhesin is greater than that of a  $\gamma$ 1 immunoadhesin.

10 With respect to the parental immunoglobulin, a useful joining point is just upstream of the cysteines of the hinge that form the disulfide bonds between the two heavy chains. In a frequently used design, the codon for the C-terminal residue of the NTN $\alpha$  part of the molecule is placed directly upstream of the codons for the sequence DKHTCPPCP of the IgG1 hinge region.

The general methods suitable for the construction and expression of immunoadhesins are the same as those disclosed hereinabove with regard to NTN $\alpha$ . NTN $\alpha$  immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the NTN $\alpha$  portion in-frame to an Ig cDNA sequence. However, fusion to genomic Ig fragments can also be used (see, e.g., Gascoigne *et al.*, *Proc. Natl. Acad. Sci. USA*, 84:2936-2940 (1987); Aruffo *et al.*, *Cell*, 61:1303-1313 (1990); Stamenkovic *et al.*, *Cell*, 66:1133-1144 (1991)). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequence from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. The cDNAs encoding the NTN $\alpha$  and Ig parts of the immunoadhesin are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells. For expression in mammalian cells, pRK5-based vectors (Schall *et al.*, *Cell*, 61:361-370 (1990)) and CDM8-based vectors (Seed, *Nature*, 329:840 (1989)) can be used. The exact junction can be created by removing the extra sequences between the designed junction codons using oligonucleotide-directed deletional mutagenesis (Zoller *et al.*, *Nucleic Acids Res.*, 10:6487 (1982); Capon *et al.*, *Nature*, 337:525-531 (1989)). Synthetic oligonucleotides can be used, in which each half is complementary to the sequence on either side of the desired junction; ideally, these are 36 to 48-mers. Alternatively, PCR techniques can be used to join the two parts of the molecule in-frame with an appropriate vector.

30 The choice of host cell line for the expression of NTN $\alpha$  immunoadhesins depends mainly on the expression vector. Another consideration is the amount of protein that is required. Milligram quantities often can be produced by transient transfections. For example, the adenovirus EIA-transformed 293 human embryonic kidney cell line can be transfected transiently with pRK5-based vectors by a modification of the calcium phosphate method to allow efficient immunoadhesin expression. CDM8-based vectors can be used to transfect COS cells by the DEAE-dextran method (Aruffo *et al.*, *Cell*, 61:1303-1313 (1990); Zettmeissl *et al.*, *DNA Cell Biol. US*, 9:347-353 (1990)). If larger amounts of protein are desired, the immunoadhesin can be expressed after stable transfection of a host cell line. For example, a pRK5-based vector can be introduced into Chinese hamster ovary (CHO) cells in the presence of an additional plasmid encoding dihydrofolate



reductase (DHFR) and conferring resistance to G418. Clones resistant to G418 can be selected in culture: these clones are grown in the presence of increasing levels of DHFR inhibitor methotrexate; clones are selected, in which the number of gene copies encoding the DHFR and immunoadhesin sequences is co-amplified. If the immunoadhesin contains a hydrophobic leader sequence at its N-terminus, it is likely to be processed and secreted by the transfected cells. The expression of immunoadhesins with more complex structures may require uniquely suited host cells; for example, components such as light chain or J chain may be provided by certain myeloma or hybridoma cell hosts (Gascoigne *et al.*, 1987, *supra*, Martin *et al.*, *J. Virol.*, 67:3561-3568 (1993)).

Immunoadhesins can be conveniently purified by affinity chromatography. The suitability of protein A as an affinity ligand depends on the species and isotype of the immunoglobulin Fc domain that is used in the chimera. Protein A can be used to purify immunoadhesins that are based on human  $\gamma 1$ ,  $\gamma 2$ , or  $\gamma 4$  heavy chains (Lindmark *et al.*, *J. Immunol. Meth.*, 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human  $\gamma 3$  (Guss *et al.*, *EMBO J.*, 5:1567-1575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. The conditions for binding an immunoadhesin to the protein A or G affinity column are dictated entirely by the characteristics of the Fc domain; that is, its species and isotype. Generally, when the proper ligand is chosen, efficient binding occurs directly from unconditioned culture fluid. One distinguishing feature of immunoadhesins is that, for human  $\gamma 1$  molecules, the binding capacity for protein A is somewhat diminished relative to an antibody of the same Fc type. Bound immunoadhesin can be efficiently eluted either at acidic pH (at or above 3.0), or in a neutral pH buffer containing a mildly chaotropic salt. This affinity chromatography step can result in an immunoadhesin preparation that is >95% pure.

Other methods known in the art can be used in place of, or in addition to, affinity chromatography on protein A or G to purify immunoadhesins. Immunoadhesins behave similarly to antibodies in thiophilic gel chromatography (Hutchens *et al.*, *Anal. Biochem.*, 159:217-226 (1986)) and immobilized metal chelate chromatography (Al-Mashikhi *et al.*, *J. Dairy Sci.*, 71:1756-1763 (1988)). In contrast to antibodies, however, their behavior on ion exchange columns is dictated not only by their isoelectric points, but also by a charge dipole that may exist in the molecules due to their chimeric nature.

If desired, the immunoadhesins can be made bispecific. Thus, the immunoadhesins of the present invention may combine a NTNR $\alpha$  extracellular domain and a domain, such as the extracellular domain, of another cytokine or neurotrophic factor receptor subunit. Exemplary cytokine receptors from which such bispecific immunoadhesin molecules can be made include TPO (or *mpl* ligand), EPO, G-CSF, IL-4, IL-7, GH, PRL, IL-3, GM-CSF, IL-5, IL-6, LIF, OSM, CNTF, GDNF and IL-2 receptors. For bispecific molecules, trimeric molecules, composed of a chimeric antibody heavy chain in one arm and a chimeric antibody heavy chain-light chain pair in the other arm of their antibody-like structure are advantageous, due to ease of purification. In contrast to antibody-producing quadromas traditionally used for the production of bispecific immunoadhesins, which produce a mixture of ten tetramers, cells transfected with nucleic acid encoding the

three chains of a trimeric immunoadhesin structure produce a mixture of only three molecules, and purification of the desired product from this mixture is correspondingly easier.

The NTN $\alpha$  protein and NTN $\alpha$  gene are believed to find *ex vivo* or *in vivo* therapeutic use for administration to a mammal, particularly humans, in the treatment of diseases or disorders, related to neurturin activity or benefited by neurturin-responsiveness. See Kotzbauer *et al. Nature* 384:467-470 (1996), which is specifically incorporated herein by reference. Conditions particularly amenable to treatment with the embodiments of the invention are those related to Ret expression or that benefit by Ret activation, particularly of the downstream pathways mediated by Ret. See Treanor *et al. Nature* 382:80-83 (1996); Jing *et al. Cell* 85:1113-1124 (1996); Trupp *et al. Nature* 381:785-789 (1996); and Durbec *et al. Nature* 381:789-793 (1996), which are specifically incorporated herein by reference. Particularly preferred are neurologic disorders, preferably central nervous system disorders, disorders of the kidney, hematopoietic disorders related to the spleen, and enteric nervous system disorders. The patient is administered an effective amount of NTN $\alpha$  protein, peptide fragment, or variant of the invention. Therapeutic methods comprising administering NTN $\alpha$ , NTN $\alpha$  agonists (e.g. NTN), NTN $\alpha$  antagonists (which compete with and bind endogenous NTN but fail to activate Ret), or anti-NTN $\alpha$  antibodies are within the scope of the present invention. The present invention also provides for pharmaceutical compositions comprising NTN $\alpha$  protein, peptide fragment, or derivative in a suitable pharmacologic carrier. The NTN $\alpha$  protein, peptide fragment, or variant may be administered systemically or locally. Applicable to the methods taught herein, the receptor protein can be optionally administered prior to, after, or preferably concomitantly with (or in complex with) NTN or other NTN $\alpha$  ligand. As taught herein, NTN $\alpha$  can be provided to target cells in the absence of NTN to increase the responsiveness of those cells to subsequently administered NTN or NTN agonist.

Certain conditions can benefit from an increase in NTN (or other NTN $\alpha$ -ligand) responsiveness. It may therefore be beneficial to increase the number of or binding affinity of NTN $\alpha$  in cells of patients suffering from such conditions. This can be achieved through administration of soluble NTN $\alpha$ , optionally complexed with NTN $\alpha$ -ligand, preferably NTN, or by gene therapy using NTN $\alpha$ -encoding nucleic acid. Selective expression of recombinant NTN $\alpha$  in appropriate cells could be achieved using NTN $\alpha$  genes controlled by tissue specific or inducible promoters or by producing localized infection with replication defective viruses carrying a recombinant NTN $\alpha$  gene. Conditions which may benefit from increased sensitivity to NTN include, but are not limited to, motoneuron disorders including amyotrophic lateral sclerosis, Werdnig-Hoffmann disease, chronic proximal spinal muscular atrophy, and Guillain-Barre syndrome. Additional conditions include those involving sympathetic neurons, particularly where increased survival or NTN-responsiveness is desired. Conditions where increased survival or NTN-responsiveness of sensory neurons, including peripheral sensory neurons, and central nervous system neurons, including dopaminergic neurons, is desirable are also suitably treated with embodiments of the invention. Accordingly, treatment of neurological disorders associated with diabetes, Parkinson's disease, Alzheimer's disease, and Huntington's chorea are provided herein. NTN finds particular use in treatment of Parkinson's disease. The present methods can also be applied to conditions related to non-neuronal cells that express NTN $\alpha$ . In fact, since NTN $\alpha$  serves to activate Ret, conditions associated with Ret-expressing cells can be treated with the embodiments of the invention.

A disease or medical disorder is considered to be nerve damage if the survival or function of nerve cells and/or their axonal processes is compromised. Such nerve damage occurs as the result conditions including (a) Physical injury, which causes the degeneration of the axonal processes and/or nerve cell bodies near the site of the injury; (b) Ischemia, as a stroke; (c) Exposure to neurotoxins, such as the cancer and AIDS chemotherapeutic agents such as cisplatin and dideoxycytidine (ddC), respectively; (d) Chronic metabolic diseases, such as diabetes or renal dysfunction; and (e) Neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Amyotrophic Lateral Sclerosis (ALS), which cause the degeneration of specific neuronal populations. Conditions involving nerve damage include Parkinson's disease, Alzheimer's disease, Amyotrophic Lateral Sclerosis, stroke, diabetic polyneuropathy, toxic neuropathy, and physical damage to the nervous system such as that caused by physical injury of the brain and spinal cord or crush or cut injuries to the arm and hand or other parts of the body, including temporary or permanent cessation of blood flow to parts of the nervous system, as in stroke.

The NTNR $\alpha$  gene is expressed in muscle cells and associated neurons. Accordingly, the present invention provides for methods of treating muscle cell disorders comprising administering to a patient in need of such treatment the compounds of the invention. Muscle cell disorders which may benefit from such treatment include but are not limited to the following progressive muscular dystrophies: Duchenne, Becker, Emery-Dreifuss, Landouzy-Dejerine, scapulohumeral, limb-girdle, Von Graefe-Fuchs, oculopharyngeal, myotonic and congenital. In addition, such molecules may be of use in the treatment of congenital (central core, nemaline, centronuclear and congenital fiber-type disproportion) and acquired (toxic, inflammatory) myopathies. The present invention further provides for a method of treating a muscle cell disorder comprising administering to the patient an effective amount of NTNR $\alpha$  protein or an active portion thereof.

In a further embodiment of the invention, patients that suffer from an excess of NTNR, hypersensitivity to NTN, excess NTN, etc. may be treated by administering an effective amount of anti-sense RNA or anti-sense oligodeoxyribonucleotides corresponding to the NTNR $\alpha$  gene coding region thereby decreasing expression of NTNR.

The compounds and methods of the invention may have use in conditions associated with a decrease in hematopoietic cells. Examples of these diseases include: anemia (including macrocytic and aplastic anemia); thrombocytopenia; hypoplasia; disseminated intravascular coagulation (DIC); myelodysplasia; immune (autoimmune) thrombocytopenic purpura (ITP); and HIV induced ITP. Additionally, these NTNR $\alpha$  molecules may be useful in treating myeloproliferative thrombocytotic diseases as well as thrombocytosis from inflammatory conditions and in iron deficiency. NTNR $\alpha$  polypeptide and NTNR $\alpha$  gene which lead to an increase in hematopoietic cell proliferation may also be used to enhance repopulation of mature blood cell lineages in cells having undergone chemo- or radiation therapy or bone marrow transplantation therapy. Generally, the NTNR $\alpha$  molecules are expected to lead to an enhancement of the proliferation and/or differentiation (but especially proliferation) of hematopoietic cells. Preferred embodiments provide for treatment to enhance hematopoiesis occurring in the spleen.

Other potential therapeutic applications for NTNR $\alpha$  and NTNR $\alpha$  gene include treatment to promote kidney or liver cell growth, survival, and repair. For example, acute renal failure refers to the abrupt disruption of previously normal kidney function. This serious clinical condition is due to a wide variety of

mechanisms including circulatory failure (shock), vascular blockage, glomerulonephritis, and obstruction to urine flow. Acute renal failure frequently arises as a complication of abdominal or vascular surgery. Also, low birth weight, high-risk neonates may now survive lung and heart problems due to continued improvements in prenatal care, only to die from complications of acute renal failure caused by infection or drug toxicity. Of particular clinical importance are cases of acute renal failure associated with trauma, sepsis, postoperative complications, or medication, particularly antibiotics. In particular, the compounds of the invention find use in etiologies, directly or indirectly, related to dysfunction of the enteric nervous system or renal system. Specific conditions affecting the GI include but are not limited to Achalasia, Esophageal spasm, Scleroderma (related to muscular atrophy of the smooth muscle portion of the esophagus, weakness of contraction of the lower two-thirds of the esophageal body, and incompetence of the lower esophageal sphincter, but also caused by treatment with immunosuppressive agents), disorders such as duodenal ulcer, Zollinger-Ellison Syndrome (hypersecretion of acid caused by factors including genetic factors, smoking, neural influences), hypersecretion of gastric acid, malabsorptive disorder for example, in diabetes (and hypoparathyroidism, hyperthyroidism, and adrenal insufficiency) where gastric atony, nausea, vomiting, etc. are at least in part related to dysfunction of the sympathetic/parasympathetic nervous system. Additional disorders include disorders of intestinal motility, including: Diverticulosis/diverticulitis; Hirschsprung's disease (a congenital disorder caused by absence of ganglion cells (Meissner's and Auerbach's plexuses) in a small segment of the distal colon, usually near the anus, typically presented in infants, but in less severe cases, may not be diagnosed until adolescence or early adulthood; Megacolon of other types (Hirschsprung's is a type of megacolon); Intestinal pseudo-obstruction, acute or chronic, which is a severe dysmotility due to abnormalities of sympathetic innervation of the muscle layers of the intestine, or secondarily may result from scleroderma, diabetes, amyloidosis, other neurologic diseases, drugs, or sepsis; chronic constipation, which is a serious problem in patients with mental retardation or neurological diseases, wherein a contributing factor is disordered gut motility. Also include are treatments for kidney diseases and disorders. Additional conditions include but not limited to: Spinal cord dysfunction, due to an obvious disruption of enteric nervous system; Guillain Barre syndrome; Multiple sclerosis; Pandy's autonomic dysfunction (dysfunction of autonomic nervous system); Parkinsonism (frequently associated with disordered gastrointestinal motility); Multiple System Atrophy (Shy Drager Syndrome), which has been documented to have as a feature disordered gut motility; and porphyria and amyloidosis which are diffuse diseases manifested by neuropathy and often with accompanying GI motility disorders.

The necrosis or damage of NTNR-expressing or NTN-responsive tissue includes a necrosis due to microbiologic infection such as viral hepatitis, tuberculosis, typhoid fever, tularemia, brucellosis, yellow fever, and the like, or necrosis due to ischemic injury resulting from shock, heart failure, and the like, or necrosis due to acute or chronic reaction with drugs and toxic substances such as chloroform, carbon tetrachloride, phosphorous poisoning, and the like. As taught herein cellular growth enhancement, including renal cells such as renal epithelial cells and neuron innervating the kidney, is useful in treating kidney disease. The compounds and methods of the present invention provide for the repair of kidney damage. Not to be bound by theory, it is believed that this can be accomplished, either directly or indirectly, by stimulating kidney cells, including innervating neurons, to grow and divide. Accordingly, a method for regenerating kidney tissue is

provided that includes the steps of preparing a NTNR $\alpha$  agonist (e.g. soluble NTNR $\alpha$  optionally complexed with NTN) as disclosed herein, optionally in combination with a pharmacologically acceptable carrier or additional growth factor or cytokine, and contacting the kidney tissue with the composition. A therapeutic amount of the composition is administered. Localized injections or implants are a preferred delivery method.

- 5 Alternatively, damaged kidneys could be removed, treated *ex vivo*, and returned to the host after the kidney is repaired.

NTNR $\alpha$  agonists, including NTN, can be administered during hemodialysis. Hemodialysis is defined as the temporary removal of blood from a patient for the purpose of extracting or separating toxins therefrom and the return of the cleansed blood to the same patient. Hemodialysis is indicated in patients where renal  
10 impairment or failure exists, that is, in cases where the blood is not being properly or sufficiently cleansed, (particularly to remove water) by the kidneys. In the case of chronic renal impairment or failure, hemodialysis has to be carried out on a repetitive basis. For example, in end stage kidney disease where transplantation of kidneys is not possible or for medical reasons is contra-indicated, the patient will have to be dialyzed about 100 to 150 times per year. This can result in several thousand accesses to the blood stream to enable the active  
15 hemodialysis to be performed over the remaining life of the patient.

The invention finds use in some immunosuppressive therapies where there is the side-effect of kidney damage. For example, therapy of IDDM in humans by methods designed to suppress the autoimmune response. Therapy utilizing cyclosporin A in diabetes can result in kidney damage. The invention finds use in disorders or conditions that can result in kidney damage. For example, diabetes can result in the typical late  
20 damages of blood vessels of the kidneys.

Other examples include immunologically- or non-immunologically-caused kidney diseases, such as e.g. glomerulonephritis, acute kidney failure, transplant rejection and kidney damage caused by nephrotic substances, kidney transplants, toxic damage to the kidneys. Furthermore, the present invention finds use in organ transplantation, including organ transport for storing any organ enucleated from a donor to insure the  
25 protection of the organ at the time of its transplantation, minimizing any trouble occurring until the transplantation operation, and to ensure the preservation of said organ in a good condition. A preferred organ is one having NTNR-bearing or NTN-responsive cells. In one specific embodiment the organ is the kidney. Use or intervention with NTNR $\alpha$  agonist, including NTN, promises success with regard to the maintenance of the kidney function.

30 As discussed herein, an object of the invention to provide methods for treatment of mammals with dysfunctional gastrointestinal muscle or disorders of smooth muscles elsewhere in the body. The gastrointestinal muscle is organized and regulated very differently than muscle elsewhere. Both skeletal and smooth muscle in the gastrointestinal tract are under the control of the enteric nervous system which is an extremely complex network of nerves and muscles, that resides within the gastrointestinal wall and  
35 orchestrates the entire digestive process including motility, secretion and absorption. The enteric nerves are also organized into interconnected networks called plexuses. Of these, the myenteric plexus, situated between the circular and longitudinal muscle layers, is the main modulator of gastrointestinal motility. It receives input from both the central nervous system (via vagal and sympathetic pathways) as well as from local reflex pathways. Its output consists of both inhibitory and excitatory signals to the adjacent muscle. The final neural

pathway regulating muscle activity in the gastrointestinal tract is therefore represented by the neurons of the myenteric plexus. A useful, if somewhat simplistic concept is to visualize net muscle tone in the gastrointestinal tract as that resulting from the balance between the opposing effects of two neuronal systems in the myenteric plexus: one causing the muscle to contract (mainly via acetylcholine) and the other causing it to relax. Both types of neurons, however, are activated by acetylcholine within the myenteric plexus. The role of acetylcholine in the regulation of gastrointestinal muscle tone is therefore complex. Acetylcholine directly released by effector nerves near the muscle causes contraction; however, within the plexus, it may result in inhibition or excitation. This is in contrast to skeletal muscle outside the gastrointestinal tract which is directly innervated by nerves emanating from the central nervous system. The interaction between nerve and muscle in skeletal muscle outside the gastrointestinal tract is far more simple: nerves release acetylcholine which causes the muscle to contract. Finally, the myenteric plexus is probably the most important but not the only determinant of muscle tone in the gastrointestinal tract. In fact, basal smooth muscle tone may be visualized as resulting from the sum of many different factors including intrinsic (myogenic) tone, and circulating hormones, in addition to nerve activity. It should be clear therefore, that the regulation of gastrointestinal tract muscle motility is far more complex than that of skeletal muscle outside the gastrointestinal tract. While there have been isolated reports on the effects of botulinum toxin on in vitro preparations of gastrointestinal smooth muscle, the regulation of gastrointestinal muscle is so complex that the physiological consequences of blocking neurotransmitter release (by using toxin such as botulinum) in humans or in live animals were not predictable prior to the present invention. The present invention provides compositions, methods, and devices for treatment of gastrointestinal disorders including achalasia, other disorders of the lower esophageal sphincter, sphincter of Oddi dysfunction, irritable bowel syndrome, and others disorders as discussed herein.

For example, provided is a method to treat Irritable Bowel Syndrome (IBS), which is a motor disorder consisting of altered bowel habits, abdominal pain, and the absence of detectable pathology. IBS is recognized by its symptoms, which are markedly influenced by psychological factors and stressful life situations. IBS is one of the most commonly encountered gastrointestinal disorders. Between 20% and 50% of patients referred to gastrointestinal clinics suffer from IBS. Symptoms of IBS occur in approximately 14% of otherwise apparently healthy people. It is a syndrome composed of a number of conditions with similar manifestations. The major symptoms of IBS (altered bowel habits, abdominal pain and bloating) are manifestations of increased motility in the gut and hyper-secretion of gastric acid. Activity of the GI tract is modulated neurally by the central nervous system (CNS) via parasympathetic and sympathetic innervation and by the peripherally located enteric nervous system (ENS) which resides within the GI tract itself.

In another aspect is provided the administration of NTNR $\alpha$  to a mammal having depressed levels of endogenous NTNR $\alpha$  or a defective NTNR $\alpha$  gene, preferably in the situation where such depressed levels lead to a pathological disorder, or where there is lack of activation of the NTNR $\alpha$  and Ret. In these embodiments where the full length NTNR $\alpha$  is to be administered to the patient, it is contemplated that the gene encoding the receptor may be administered to the patient via gene therapy technology.

In gene therapy applications, genes are introduced into cells in order to achieve *in vivo* synthesis of a therapeutically effective genetic product, for example for replacement of a defective gene. "Gene therapy"

includes both conventional gene therapy where a lasting effect is achieved by a single treatment, and the administration of gene therapeutic agents, which involves the one time or repeated administration of a therapeutically effective DNA or mRNA. Antisense RNAs and DNAs can be used as therapeutic agents for blocking the expression of certain genes *in vivo*. It has already been shown that short antisense oligonucleotides can be imported into cells where they act as inhibitors, despite their low intracellular concentrations caused by their restricted uptake by the cell membrane. (Zamecnik *et al.*, *Proc. Natl. Acad. Sci. USA*, 83:4143-4146 (1986)). The oligonucleotides can be modified to enhance their uptake, *e.g.*, by substituting their negatively charged phosphodiester groups by uncharged groups.

There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells *in vitro*, *ex vivo*, or *in vivo* in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. The currently preferred *in vivo* gene transfer techniques include transfection with viral (typically retroviral) vectors and viral coat protein-liposome mediated transfection (Dzau *et al.*, *Trends in Biotechnology*, 11:205-210 (1993)). In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein or the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, *e.g.* capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, and proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu *et al.*, *J. Biol. Chem.*, 262:4429-4432 (1987); and Wagner *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:3410-3414 (1990). For review of the currently known gene marking and gene therapy protocols see Anderson *et al.*, *Science*, 256:808-813 (1992).

The invention also provides antagonists of NTNR $\alpha$  activation (*e.g.*, NTNR $\alpha$  antisense nucleic acid, neutralizing antibodies). Administration of NTNR $\alpha$  antagonist to a mammal having increased or excessive levels of endogenous NTNR $\alpha$  activation is contemplated, preferably in the situation where such increased levels of NTNR $\alpha$  or Ret activation lead to a pathological disorder.

In one embodiment, NTNR $\alpha$  antagonist molecules may be used to bind endogenous ligand in the body, thereby causing desensitized NTNR $\alpha$  to become responsive to NTN ligand, especially when the levels of NTN ligand in the serum exceed normal physiological levels. Also, it may be beneficial to bind endogenous NTN ligand which is activating undesired cellular responses (such as proliferation of tumor cells).

Pharmaceutical compositions of the soluble NTNR $\alpha$  can further include a NTN or other NTNR $\alpha$  agonist. Such dual compositions may be beneficial where it is therapeutically useful to prolong half-life of NTN, provide a slow-release reservoir for NTN, activate endogenous NTNR $\alpha$  or Ret, and/or to supplement the lack of NTNR $\alpha$  in a target Ret-expressing cell, thereby rendering the cell responsive to NTN.

Therapeutic formulations of NTNR $\alpha$  are prepared for storage by mixing NTNR $\alpha$  having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (*Remington's Pharmaceutical Sciences*, 16th edition, Osol, A., Ed., (1980)), in the form of lyophilized cake or aqueous

solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counter-ions such as sodium; and/or non-ionic surfactants such as Tween, Pluronics or polyethylene glycol (PEG).

The NTN $\alpha$  also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles, and nanocapsules), or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences, supra*.

NTN $\alpha$  to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. NTN $\alpha$  ordinarily will be stored in lyophilized form or in solution.

Therapeutic NTN $\alpha$  compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The route of NTN $\alpha$  administration is in accord with known methods, *e.g.*, those routes set forth above for specific indications, as well as the general routes of injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional means, or sustained release systems as noted below. NTN $\alpha$  is administered continuously by infusion or by bolus injection. Generally, where the disorder permits, one should formulate and dose the NTN $\alpha$  for site-specific delivery. Administration can be continuous or periodic. Administration can be accomplished by a constant- or programmable-flow implantable pump or by periodic injections.

Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the protein, which matrices are in the form of shaped articles, *e.g.*, films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (*e.g.*, poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, *J. Biomed. Mater. Res.*, 15:167-277 (1981) and Langer, *Chem. Tech.*, 12:98-105 (1982) or poly(vinylalcohol)), polylactides (U.S. Patent No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and  $\gamma$  ethyl-L-glutamate (Sidman *et al.*, *Biopolymers*, 22:547-556 (1983)), non-degradable ethylene-vinyl acetate (Langer *et al.*, *supra*), degradable lactic acid-glycolic acid copolymers such as the Lupron Depot<sup>TM</sup> (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988).

While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated proteins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for protein stabilization depending on the mechanism involved. For example, if the



aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

5 Sustained-release NTNR $\alpha$  compositions also include liposomally entrapped NTNR $\alpha$ . Liposomes containing NTNR $\alpha$  are prepared by methods known *per se*: DE 3,218,121; Epstein *et al.*, *Proc. Natl. Acad. Sci. USA*, 82:3688-3692 (1985); Hwang *et al.*, *Proc. Natl. Acad. Sci. USA*, 77:4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. Patent Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800  
10 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal NTNR $\alpha$  therapy.

When applied topically, the NTNR $\alpha$  is suitably combined with other ingredients, such as carriers and/or adjuvants. There are no limitations on the nature of such other ingredients, except that they must be physiologically acceptable and efficacious for their intended administration, and cannot degrade the activity  
15 of the active ingredients of the composition. Examples of suitable vehicles include ointments, creams, gels, or suspensions, with or without purified collagen. The compositions also may be impregnated into transdermal patches, plasters, and bandages, preferably in liquid or semi-liquid form.

For obtaining a gel formulation, the NTNR $\alpha$  formulated in a liquid composition may be mixed with an effective amount of a water-soluble polysaccharide or synthetic polymer such as PEG to form a gel of the  
20 proper viscosity to be applied topically. The polysaccharide that may be used includes, for example, cellulose derivatives such as etherified cellulose derivatives, including alkyl celluloses, hydroxyalkyl celluloses, and alkylhydroxyalkyl celluloses, for example, methylcellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methylcellulose, and hydroxypropyl cellulose; starch and fractionated starch; agar; alginic acid and alginates; gum arabic; pullullan; agarose; carrageenan; dextrans; dextrans; fructans; inulin; mannans;  
25 xylans; arabinans; chitosans; glycogens; glucans; and synthetic biopolymers; as well as gums such as xanthan gum; guar gum; locust bean gum; gum arabic; tragacanth gum; and karaya gum; and derivatives and mixtures thereof. The preferred gelling agent herein is one that is inert to biological systems, nontoxic, simple to prepare, and not too runny or viscous, and will not destabilize the NTNR $\alpha$  held within it.

Preferably the polysaccharide is an etherified cellulose derivative, more preferably one that is well  
30 defined, purified, and listed in USP, *e.g.*, methylcellulose and the hydroxyalkyl cellulose derivatives, such as hydroxypropyl cellulose, hydroxyethyl cellulose, and hydroxypropyl methylcellulose. Most preferred herein is methylcellulose.

The polyethylene glycol useful for gelling is typically a mixture of low and high molecular weight PEGs to obtain the proper viscosity. For example, a mixture of a PEG of molecular weight 400-600 with one  
35 of molecular weight 1500 would be effective for this purpose when mixed in the proper ratio to obtain a paste.

The term "water soluble" as applied to the polysaccharides and PEGs is meant to include colloidal solutions and dispersions. In general, the solubility of the cellulose derivatives is determined by the degree of substitution of ether groups, and the stabilizing derivatives useful herein should have a sufficient quantity of such ether groups per anhydroglucose unit in the cellulose chain to render the derivatives water soluble.

A degree of ether substitution of at least 0.35 ether groups per anhydroglucose unit is generally sufficient. Additionally, the cellulose derivatives may be in the form of alkali metal salts, for example, the Li, Na, K, or Cs salts.

If methylcellulose is employed in the gel, preferably it comprises about 2-5%, more preferably about 3%, of the gel and the NTN $\alpha$  is present in an amount of about 300-1000 mg per ml of gel.

Semipermeable, implantable membrane devices are useful as means for delivering drugs in certain circumstances. For example, cells that secrete soluble NTN $\alpha$  or chimeras can be encapsulated, and such devices can be implanted into a patient. For example, into the brain of patients suffering from Parkinson's Disease. See, U.S. Patent No. 4,892,538 of Aebischer *et al.*; U.S. Patent No. 5,011,472 of Aebischer *et al.*; U.S. Patent No. 5,106,627 of Aebischer *et al.*; PCT Application WO 91/10425; PCT Application WO 91/10470; Winn *et al.*, *Exper. Neurology*, 113:322-329 (1991); Aebischer *et al.*, *Exper. Neurology*, 111:269-275 (1991); and Tresco *et al.*, *ASAIO*, 38:17-23 (1992). Accordingly, also included is a method for preventing or treating damage to a nerve or damage to other NTN $\alpha$ -expressing or NTN-responsive cells, e.g. kidney, as taught herein, which comprises implanting cells that secrete NTN $\alpha$ , its agonists or antagonists as may be required for the particular condition, into the body of patients in need thereof. Finally, the present invention includes a device for preventing or treating nerve damage or damage to other cells as taught herein by implantation into a patient comprising a semipermeable membrane, and a cell that secretes NTN $\alpha$  (or its agonists or antagonists as may be required for the particular condition) encapsulated within said membrane and said membrane being permeable to NTN $\alpha$  (or its agonists or antagonists) and impermeable to factors from the patient detrimental to the cells. The patient's own cells, transformed to produce NTN or NTN $\alpha$  *ex vivo*, could be implanted directly into the patient, optionally without such encapsulation. The methodology for the membrane encapsulation of living cells is familiar to those of ordinary skill in the art, and the preparation of the encapsulated cells and their implantation in patients may be accomplished without under experimentation.

The present invention includes, therefore, a method for preventing or treating nerve damage by implanting cells, into the body of a patient in need thereof, cells either selected for their natural ability to generate NTN $\alpha$  or engineered to secrete NTN $\alpha$ . Preferably, the secreted NTN $\alpha$  being soluble, human mature NTN $\alpha$  when the patient is human. The implants are preferably non-immunogenic and/or prevent immunogenic implanted cells from being recognized by the immune system. For CNS delivery, a preferred location for the implant is the cerebral spinal fluid of the spinal cord.

An effective amount of NTN $\alpha$  to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. Typically, the clinician will administer the NTN $\alpha$  until a dosage is reached that achieves the desired effect. A typical daily dosage for systemic treatment might range from about 1  $\mu$ g/kg to up to 10 mg/kg or more, depending on the factors mentioned above. As an alternative general proposition, the NTN $\alpha$  is formulated and delivered to the target site or tissue at a dosage capable of establishing in the tissue a NTN $\alpha$  level greater than about 0.1 ng/cc up to a maximum dose that is efficacious but not unduly toxic. This intra-tissue concentration should be maintained if possible by continuous infusion, sustained

release, topical application, NTN $\alpha$ -expressing cell implant, or injection at empirically determined frequencies. The progress of this therapy is easily monitored by conventional assays. When administered in complex with or concomitantly with NTN, a 100:1 to 1:100 ratio of NTN $\alpha$  to NTN dimer is useful. Preferably the ratio is 10:1 to 1:10, more preferably 1:1, and even more preferably 2:1, which may reflect the natural binding ratio of NTN $\alpha$  to NTN. An amount of NTN effective to produce the desired result is administered. A typical daily dosage for systemic treatment might also range from about 1  $\mu$ g/kg to up to 10 mg/kg or more, depending on the factors mentioned above.

NTN $\alpha$  nucleic acid is useful for the preparation of NTN $\alpha$  polypeptide by recombinant techniques exemplified herein which can then be used for production of anti-NTN $\alpha$  antibodies having various utilities described below.

The NTN $\alpha$  (polypeptide or nucleic acid) can be used to increase NTN-responsiveness (and thus increase cell survival and modulate Ret-mediated downstream pathways) of cells *in vitro*. Such cells must contain or be modified to contain cell surface Ret. Cultured *ex vivo*, these cells may simultaneously be exposed to other known neurotrophic factors or cytokines, such as those described herein.

In yet another aspect of the invention, the NTN $\alpha$  may be used for affinity purification of ligands that bind to the NTN $\alpha$ , either naturally-occurring or synthetic ligands. NTN is a preferred ligand for purification. Briefly, this technique involves: (a) contacting a source of NTN ligand with an immobilized NTN $\alpha$  under conditions whereby the NTN ligand to be purified is selectively adsorbed onto the immobilized receptor; (b) washing the immobilized NTN $\alpha$  and its support to remove non-adsorbed material; and (c) eluting the NTN ligand molecules from the immobilized NTN $\alpha$  to which they are adsorbed with an elution buffer. In a particularly preferred embodiment of affinity purification, NTN $\alpha$  is covalently attaching to an inert and porous matrix or resin (*e.g.*, agarose reacted with cyanogen bromide). Especially preferred is a NTN $\alpha$  immunoadhesin immobilized on a protein A column. A solution containing NTN ligand is then passed through the chromatographic material. The NTN ligand adsorbs to the column and is subsequently released by changing the elution conditions (*e.g.* by changing pH or ionic strength). Novel ligands can be detected by monitoring displacement of a known, labelled NTN $\alpha$  ligand, such as  $^{125}$ I- or biotinylated-NTN.

The NTN $\alpha$  may be used for competitive screening of potential agonists or antagonists for binding to the NTN $\alpha$ . Such agonists or antagonists may constitute potential therapeutics for treating conditions characterized by insufficient or excessive NTN $\alpha$  activation, respectively.

The preferred technique for identifying molecules which bind to the NTN $\alpha$  utilizes a chimeric receptor (*e.g.*, epitope-tagged NTN $\alpha$  or NTN $\alpha$  immunoadhesin) attached to a solid phase, such as the well of an assay plate. The binding of the candidate molecules, which are optionally labelled (*e.g.*, radiolabeled), to the immobilized receptor can be measured. Alternatively, competition for binding of a known, labelled NTN $\alpha$  ligand, such as  $^{125}$ I-NTN, can be measured. For screening for antagonists, the NTN $\alpha$  can be exposed to a NTN ligand followed by the putative antagonist, or the NTN ligand and antagonist can be added to the NTN $\alpha$  simultaneously, and the ability of the antagonist to block receptor activation can be evaluated.

The present invention also provides for assay systems for detecting NTN activity, comprising cells which express high levels of NTN $\alpha$ , and which are, therefore, extremely sensitive to even very low concentrations of NTN or NTN-like molecules. The present invention provides for assay systems in which

NTN activity or activities similar to NTN activity resulting from exposure to a peptide or non-peptide compound may be detected by measuring a physiological response to NTN in a cell or cell line responsive to NTN which expresses the NTNR $\alpha$  molecules of the invention. A physiological response may comprise any of the biological effects of NTN, including but not limited to, those described herein, as well as the transcriptional activation of certain nucleic acid sequences (e.g. promoter/enhancer elements as well as structural genes), NTN-related processing, translation, or phosphorylation, the induction of secondary processes in response to processes directly or indirectly induced by NTN, including Ret-mediated effects, and morphological changes, such as neurite sprouting, or the ability to support the survival of cells, for example, nodose or dorsal root ganglion cells, motoneurons, dopaminergic neurons, sensory neurons, Purkinje cells, or hippocampal neurons.

In one embodiment of the invention, the functional interaction between NTN and the NTNR $\alpha$  may be observed by detecting an increase in the production autophosphorylated Ret protein, or alternatively, phosphorylated ERK-1 or ERK-2 homologs (See Kotzbauer *et al.*, *supra*).

The present invention provides for the development of novel assay systems which can be utilized in the screening of compounds for NTN- or NTN-like activity. Target cells which bind NTN may be produced by transfection with NTNR $\alpha$ -encoding nucleic acid or may be identified and segregated by, for example, fluorescent-activated cell sorting, sedimentation of rosettes, or limiting dilution. Once target cell lines are produced or identified, it may be desirable to select for cells which are exceptionally sensitive to NTN. Such target cells may bear a greater number of NTNR $\alpha$  molecules; target cells bearing a relative abundance of NTNR $\alpha$  can be identified by selecting target cells which bind to high levels of NTN, for example, by marking high-expressors with fluorophore tagged-NTN followed by immunofluorescence detection and cell sorting. Alternatively, cells which are exceptionally sensitive to NTN may exhibit a relatively strong biological response in response to NTN binding, such as a sharp increase in Ret-mediated effects or in immediate early gene products such as c-fos or c-jun. By developing assay systems using target cells which are extremely sensitive to NTN, the present invention provides for methods of screening for NTN or NTN-like activity which are capable of detecting low levels of NTN activity.

In particular, using recombinant DNA techniques, the present invention provides for NTN target cells which are engineered to be highly sensitive to NTN. For example, the NTN-receptor gene can be inserted into cells which are naturally NTN responsive such that the recombinant NTNR $\alpha$  gene is expressed at high levels and the resulting engineered target cells express a high number of NTNRs on their cell surface. Alternatively, or additionally, the target cells may be engineered to comprise a recombinant gene which is expressed at high levels in response to NTN/receptor binding. Such a recombinant gene may preferably be associated with a readily detectable product. For example, and not by way of limitation, transcriptional control regions (i.e. promoter/enhancer regions) from an immediate early gene may be used to control the expression of a reporter gene in a construct which may be introduced into target cells. The immediate early gene/reporter gene construct, when expressed at high levels in target cells by virtue of a strong promoter/enhancer or high copy number, may be used to produce an amplified response to NTNR $\alpha$  binding. For example, and not by way of limitation, a NTN-responsive promoter may be used to control the expression of detectable reporter genes including  $\beta$ -galactosidase, growth hormone, chloramphenicol acetyl transferase, neomycin phosphotransferase,

luciferase, or  $\beta$ -glucuronidase. Detection of the products of these reporter genes, well known to one skilled in the art, may serve as a sensitive indicator for NTN or NTN-like activity of pharmaceutical compounds.

The NTNR $\alpha$ -encoding or reporter gene constructs discussed herein (e.g., soluble ECD) can be inserted into target cells using any method known in the art, including but not limited to transfection, electroporation, calcium phosphate/DEAE dextran methods, and cell gun. The constructs and engineered target cells can be used for the production of transgenic animals bearing the above-mentioned constructs as transgenes, from which NTNR $\alpha$ -expressing target cells may be selected using the methods discussed.

Nucleic acids which encode NTNR, preferably from non-human species, such as murine or rat protein, can be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal (e.g., a mouse) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic, stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, the human and/or rat cDNA encoding NTNR $\alpha$ , or an appropriate sequence thereof, can be used to clone genomic DNA encoding NTNR $\alpha$  in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding NTNR. Methods for generating transgenic animals, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for NTNR $\alpha$  transgene incorporation with tissue-specific enhancers, which could result in desired effect of treatment. Transgenic animals that include a copy of a transgene encoding NTNR $\alpha$  introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding NTNR. Such animals can be used as tester animals for reagents thought to confer protection from, for example, diseases related to NTN. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the disease, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the disease.

Transgenic mice bearing minigenes are currently preferred. First a fusion enzyme expression construct is created and selected based on expression in cell culture as described in the Examples. Then a minigene capable of expressing that fusion enzyme is constructed using known techniques. Particularly preferred hosts are those bearing minigene constructs comprising a transcriptional regulatory element that is tissue-specific for expression.

Transgenic mice expressing NTNR $\alpha$  minigene are made using known techniques, involving, for example, retrieval of fertilized ova, microinjection of the DNA construct into male pronuclei, and re-insertion of the fertilized transgenic ova into the uteri of hormonally manipulated pseudopregnant foster mothers. Alternatively, chimeras are made using known techniques employing, for example, embryonic stem cells (Rossant *et al.*, *Philos. Trans. R. Soc. Lond. Biol.* 339:207-215 (1993)) or primordial germ cells (Vick *et al.*, *Philos. Trans. R. Soc. Lond. Biol.* 251:179-182 (1993)) of the host species. Insertion of the transgene can be evaluated by Southern blotting of DNA prepared from the tails of offspring mice. Such transgenic mice are then back-crossed to yield homozygotes.

It is now well-established that transgenes are expressed more efficiently if they contain introns at the 5' end, and if these are the naturally occurring introns (Brinster *et al.* *Proc. Natl. Acad. Sci. USA* 85:836 (1988); Yokode *et al.*, *Science* 250:1273 (1990)).

Transgenic mice expressing NTNR $\alpha$  minigene are created using established procedures for creating transgenic mice. Transgenic mice are constructed using now standard methods ( *et al.* *Proc. Natl. Acad. Sci. USA* 85:836 (1988); Yokode *et al.*, *Science* 250:1273 (1990); Rubin *et al.*, *Proc Natl Acad Sci USA* 88:434 (1991); Rubin *et al.* *Nature* 353:265 (1991)). Fertilized eggs from timed matings are harvested from the oviduct by gentle rinsing with PBS and are microinjected with up to 100 nanoliters of a DNA solution, delivering about 10<sup>4</sup> DNA molecules into the male pronucleus. Successfully injected eggs are then re-implanted into pseudopregnant foster mothers by oviduct transfer. Less than 5% of microinjected eggs yield transgenic offspring and only about 1/3 of these actively express the transgene: this number is presumably influenced by the site at which the transgene enters the genome.

Transgenic offspring are identified by demonstrating incorporation of the microinjected transgene into their genomes, preferably by preparing DNA from short sections of tail and analyzing by Southern blotting for presence of the transgene ("Tail Blots"). A preferred probe is a segment of a minigene fusion construct that is uniquely present in the transgene and not in the mouse genome. Alternatively, substitution of a natural sequence of codons in the transgene with a different sequence that still encodes the same peptide yields a unique region identifiable in DNA and RNA analysis. Transgenic "founder" mice identified in this fashion are bred with normal mice to yield heterozygotes, which are back-crossed to create a line of transgenic mice. Tail blots of each mouse from each generation are examined until the strain is established and homozygous. Each successfully created founder mouse and its strain vary from other strains in the location and copy number of transgenes inserted into the mouse genome, and hence have widely varying levels of transgene expression. Selected animals from each established line are sacrificed at 2 months of age and the expression of the transgene is analyzed by Northern blotting of RNA from liver, muscle, fat, kidney, brain, lung, heart, spleen, gonad, adrenal and intestine.

Alternatively, the non-human homologs of NTNR $\alpha$  can be used to construct a NTNR $\alpha$  "knock out" animal, i.e., having a defective or altered gene encoding NTNR, as a result of homologous recombination between the endogenous NTNR $\alpha$  gene and an altered genomic NTNR $\alpha$  DNA introduced into an embryonic cell of the animal. For example, murine NTNR $\alpha$  cDNA can be used to clone genomic NTNR $\alpha$  DNA in accordance with established techniques. A portion of the genomic NTNR $\alpha$  DNA (e.g., such as an exon which encodes e.g., an extracellular domain) can be deleted or replaced with another gene, such as a gene encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector (see e.g., Thomas and Capecchi, *Cell* 51:503 (1987) for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected (see e.g., Li *et al.*, *Cell* 69: 915 (1992)). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse) to form aggregation chimeras (see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster

animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for their ability to accept grafts, reject tumors and defend against infectious diseases and can be used in the study of basic immunobiology.

In addition to the above procedures, which can be used for preparing recombinant DNA molecules and transformed host animals in accordance with the practices of this invention, other known techniques and modifications thereof can be used in carrying out the practice of the invention. For example, U.S. Pat. No. 4,736,866 discloses vectors and methods for production of a transgenic non-human eukaryotic animal whose germ cells and somatic cells contain a gene sequence introduced into the animal, or an ancestor of the animal, at an embryonic stage. U.S. Pat. No. 5,087,571 discloses a method of providing a cell culture comprising (1) providing a transgenic non-human mammal, all of whose germ cells and somatic cells contain a recombinant gene sequence introduced at an embryonic stage; and (2) culturing one or more of said somatic cells. U.S. Pat. No. 5,175,385 discloses vectors and methods for production of a transgenic mouse whose somatic and germ cells contain and express a gene at sufficient levels to provide the desired phenotype in the mouse, the gene having been introduced into said mouse or an ancestor of said mouse at an embryonic stage, preferably by microinjection. A partially constitutive promoter, the metallothionein promoter, was used to drive heterologous gene expression. U.S. Pat. No. 5,175,384 discloses a method of introducing a transgene into an embryo by infecting the embryo with a retrovirus containing the transgene. U.S. Pat. No. 5,175,383 discloses DNA constructs having a gene, homologous to the host cell, operably linked to a heterologous and inducible promoter effective for the expression of the gene in the urogenital tissues of a mouse, the transgene being introduced into the mouse at an embryonic stage to produce a transgenic mouse. Even though a homologous gene is introduced, the gene can integrate into a chromosome of the mouse at a site different from the location of the endogenous coding sequence. The vital MMTV promoter was disclosed as a suitable inducible promoter. U.S. Pat. No. 5,162,215 discloses methods and vectors for transfer of genes in avian species, including livestock species such as chickens, turkeys, quails or ducks, utilizing pluripotent stem cells of embryos to produce transgenic animals. U.S. Pat. No. 5,082,779 discloses pituitary-specific expression promoters for use in producing transgenic animals capable of tissue-specific expression of a gene. U.S. Pat. No. 5,075,229 discloses vectors and methods to produce transgenic, chimeric animals whose hemopoietic liver cells contain and express a functional gene driven by a liver-specific promoter, by injecting into the peritoneal cavity of a host fetus the disclosed vectors such that the vector integrates into the genome of fetal hemopoietic liver cells.

Although some of the above-mentioned patents and publications are directed to the production or use of a particular gene product or material that are not within the scope of the present invention, the procedures described therein can easily be modified to the practice of the invention described in this specification by those skilled in the art of fermentation and genetic engineering.

Assay systems of the present invention enable the efficient screening of pharmaceutical compounds for use in the treatment of NTN-associated diseases. For example, and not by way of limitation, it may be desirable to screen a pharmaceutical agent for NTN activity and therapeutic efficacy in cerebellar

degeneration. In a one embodiment of the invention, cells responsive to NTN may be identified and isolated, and then cultured in microwells in a multiwell culture plate. Culture medium with added test agent, or added NTN, in numerous dilutions may be added to the wells, together with suitable controls. The cells may then be examined for improved survival, neurite sprouting, and the like, and the activity of test agent and NTN, as well as their relative activities, can be determined. For example, one can now identify NTN-like compounds which can, like NTN, prevent motoneuron cell death in response to toxic assault or axotomy, for example. NTN responsive motoneurons could be utilized in assay systems to identify compounds useful in treating motoneuron diseases. If a particular disease is found to be associated with a defective NTN response in a particular tissue, a rational treatment for the disease would be supplying the patient with exogenous NTN. However, it may be desirable to develop molecules which have a longer half-life than endogenous NTN, or which act as NTN agonists, or which are targeted to a particular tissue. Accordingly, the methods of the invention can be used to produce efficient and sensitive screening systems which can be used to identify molecules with the desired properties. Similar assay systems could be used to identify NTN antagonists.

In addition, the present invention provides for experimental model systems for studying the physiological role of NTN and its receptor. Such systems include animal models, such as (i) animals exposed to circulating NTN $\alpha$  peptides which compete with cellular receptor for NTN binding and thereby produce a NTN-depleted condition, (ii) animals immunized with NTN $\alpha$ ; (iii) transgenic animals which express high levels of NTN $\alpha$  and therefore are hypersensitive to NTN; and (iv) animals derived using embryonic stem cell technology in which the endogenous NTN $\alpha$  genes were deleted from the genome.

The present invention also provides for experimental model systems for studying the physiological role of NTN and its receptor. In these model systems NTN $\alpha$  protein, peptide fragment, or a derivative thereof, may be either supplied to the system or produced within the system. Such model systems could be used to study the effects of NTN excess or NTN depletion. The experimental model systems may be used to study the effects of increased or decreased response to NTN in cell or tissue cultures, in whole animals, in particular cells or tissues within whole animals or tissue culture systems, or over specified time intervals (including during embryogenesis) in embodiments in which NTN $\alpha$  expression is controlled by an inducible or developmentally regulated promoter. In a particular embodiment of the invention, the CMV promoter may be used to control expression of NTN $\alpha$  in transgenic animals. Transgenic animals, as discussed herein, are produced by any method known in the art, including, but not limited to microinjection, cell fusion, transfection, and electroporation.

The present invention also provides for model systems for autoimmune disease in which an autoimmune response is directed toward NTN $\alpha$ . Such models comprise animals which have been immunized with immunogenic amounts of NTN $\alpha$  and preferably found to produce anti-NTN $\alpha$  antibodies and/or cell-mediated immunity. To produce such a model system, it may be desirable to administer the NTN $\alpha$  in conjunction with an immune adjuvant.

For example, and not by way of limitation, an experimental model system may be created which may be used to study the effects of excess NTN activity. In such a system, the response to NTN may be increased by engineering an increased number of NTN $\alpha$ s on cells of the model system relative to cells which have not been so engineered. These cells should also express Ret or another signalling molecule capable of interacting



with NTNR $\alpha$  and mediating an NTN signal. It may be preferable to provide an increased number of NTNRs selectively on cells which normally express NTNRs. Cells may be engineered to produce increased numbers of NTNR $\alpha$  by infection with a virus which carries a NTNR $\alpha$  gene of the invention. Alternatively, the NTNR $\alpha$  gene may be provided to the cells by transfection. If the model system is an animal, a recombinant NTNR $\alpha$  gene may be introduced into the cells of the animal by infection with a virus which carries the NTNR $\alpha$  gene or other means as discussed herein. For example, a transgenic animal may be created which carries the NTNR $\alpha$  gene as a transgene. In order to ensure expression of NTNR, the NTNR $\alpha$  gene should be placed under the control of a suitable promoter sequence. It may be desirable to put the NTNR $\alpha$  gene under the control of a constitutive and/or tissue specific promoter. By increasing the number of cellular NTNRs, the response to endogenous NTN may be increased. If the model system contains little or no NTN, NTN may be added to the system. It may also be desirable to add additional NTN to the model system in order to evaluate the effects of excess NTN activity. Over expressing NTN (or secreted NTN) may be the preferable method for studying the effects of elevated levels of NTN on cells already expressing NTNR. More preferably would be to express NTNR $\alpha$  in all cells (general expression) and determine which cells are then endowed with functional responsiveness to NTN, thus allowing the potential identification of a second receptor component, if one exists.

An experimental model system may be created which may be used to study the effects of diminished NTN activity. This system may permit identification of processes or neurons which require NTN, and which may represent potential therapeutic targets. In such a system, the response to NTN may be decreased by providing recombinant NTNRs which are not associated with a cell surface or which are engineered so as to be ineffective in transducing a response to NTN. For example, NTNR $\alpha$  protein, peptide, or derivative may be supplied to the system such that the supplied receptor may compete with endogenous NTNR $\alpha$  for NTN binding, thereby diminishing the response to NTN. The NTNR $\alpha$  may be a cell free receptor which is either added to the system or produced by the system. For example, a NTNR $\alpha$  protein which lacks the transmembrane domain may be produced by cells within the system, such as an anchorless NTNR $\alpha$  that may be secreted from the producing cell. Alternatively, NTNR $\alpha$  protein, peptide or derivative may be added to an extracellular space within the system. In additional embodiments of the invention, a recombinant NTNR $\alpha$  gene may be used to inactivate or "knock out" the endogenous gene by homologous recombination, and thus create a NTNR $\alpha$  deficient cell, tissue, or animal. For example, and not by way of limitation, a recombinant NTNR $\alpha$  gene may be engineered to contain an insertional mutation, for example the neo gene, which inactivates NTNR. Such a construct, under the control of a suitable promoter, may be introduced into a cell, such as an embryonic stem cell, by a technique such as transfection, transduction, injection, etc. Cells containing the construct may then be selected by G418 resistance. Cells which lack an intact NTNR $\alpha$  gene may then be identified, e.g. by Southern blotting or Northern blotting or assay of expression. Cells lacking an intact NTNR $\alpha$  gene may then be fused to early embryo cells to generate transgenic animals deficient in NTNR. A comparison of such an animal with an animal not expressing endogenous NTN would reveal that either the two phenotypes match completely or that they do not, implying the presence of additional NTN-like factors or receptors. Such an animal may be used to define specific cell populations, e.g., neuronal populations, or any other *in vivo* processes, normally dependent upon NTN or its receptor. Thus, these

populations or processes may be expected to be effected if the animal did not express NTNR $\alpha$  and therefore could not respond to NTN. Alternatively, a recombinant NTNR $\alpha$  protein, peptide, or derivative which competes with endogenous receptor for NTN may be expressed on the surface of cells within the system, but may be engineered so as to fail to transduce a response to NTN binding. The recombinant NTNR $\alpha$  proteins, peptides or derivatives described above may bind to NTN with an affinity that is similar to or different from the affinity of endogenous NTNR $\alpha$  to NTN. To more effectively diminish the response to NTN, the NTNR $\alpha$  protein, peptide, or derivative may desirably bind to NTN with a greater affinity than that exhibited by the native receptor. If the NTNR $\alpha$  protein, peptide, or derivative is produced within the model system, nucleic acid encoding the NTNR $\alpha$  protein, peptide, or derivative may be supplied to the system by infection, transduction, transfection, etc. or as a transgene. As discussed supra, the NTNR $\alpha$  gene may be placed under the control of a suitable promoter, which may be, for example, a tissue-specific promoter or an inducible promoter or developmentally regulated promoter. In a specific embodiment of the invention the endogenous NTNR $\alpha$  gene of a cell may be replaced by a mutant NTNR $\alpha$  gene by homologous recombination. In a further embodiment of the invention, NTNR $\alpha$  expression may be reduced by providing NTNR $\alpha$  expressing cells with an amount of NTNR $\alpha$  antisense RNA or DNA effective to reduce expression of NTNR $\alpha$  protein.

The polypeptides of the invention also find use as feed additives for animals. The nucleic acids of the invention find use in preparing these polypeptides.

The NTNR $\alpha$  polypeptides are also useful as molecular weight markers. To use a NTNR $\alpha$  polypeptide as a molecular weight marker, gel filtration chromatography or SDS-PAGE, for example, will be used to separate protein(s) for which it is desired to determine their molecular weight(s) in substantially the normal way. NTNR $\alpha$ , preferably a soluble NTNR, and other molecular weight markers will be used as standards to provide a range of molecular weights. For example, phosphorylase b (mw = 97,400), bovine serum albumin (mw = 68,000), ovalbumin (mw = 46,000), trypsin inhibitor (mw = 20,100), and lysozyme (mw = 14,400) can be used as MW markers. The other molecular weight markers mentioned here can be purchased commercially from Amersham Corporation, Arlington Heights, IL. The molecular weight markers are generally labeled to facilitate detection thereof. For example, the markers may be biotinylated and, following separation, can be incubated with streptavidin-horseradish peroxidase so that the various markers can be detected by light detection.

The purified NTNR $\alpha$ , and the nucleic acid encoding it, may also be sold as reagents for mechanism studies of NTNR $\alpha$  and its ligands, to study the role of the NTNR $\alpha$  and NTN ligand in normal growth and development, as well as abnormal growth and development, *e.g.*, in malignancies. NTNR $\alpha$  probes can be used to identify cells and tissues which are responsive to NTN in normal or diseased states. For example, a patient suffering from a NTN-related disorder may exhibit an aberrancy of NTNR $\alpha$  expression. The present invention provides for methods for identifying cells which are responsive to NTN comprising detecting NTNR $\alpha$  expression in such cells. NTNR $\alpha$  expression may be evidenced by transcription of NTNR $\alpha$  mRNA or production of NTNR $\alpha$  protein. NTNR $\alpha$  expression may be detected using probes which identify NTNR $\alpha$  nucleic acid or protein. One variety of probe which may be used to detect NTNR $\alpha$  expression is a nucleic acid probe, which may be used to detect NTNR-encoding RNA by any method known in the art, including,

but not limited to, in situ hybridization, Northern blot analysis, or PCR related techniques. Another variety of probe which may be used is tagged NTN as discussed herein.

According to the invention, tagged NTN may be incubated with cells under conditions which would promote the binding or attachment of NTN to said cells. In most cases, this may be achieved under standard culture conditions. For example, in one embodiment of the invention, cells may be incubated for about 30 minutes in the presence of tagged NTN. If the tag is an antibody molecule, it may be preferable to allow NTN to bind to cells first and subsequently wash cells to remove unbound ligand and then add anti-NTN antibody tag. In another embodiment of the invention, tagged NTN on the surface of NTN-responsive cells, hereafter called target cells, may be detected by rosetting assays in which indicator cells that are capable of binding to the tag are incubated with cells bearing tagged-NTN such that they adhere to tagged-NTN on the target cells and the bound indicator cells form rosette-like clusters around NTN-tag bearing cells. These rosettes may be visualized by standard microscopic techniques on plated cells, or, alternatively, may allow separation of rosetted and non-rosetted cells by density centrifugation. In a preferred specific embodiment of the invention, target cells, such as neuronal cells. In alternative embodiments of the invention, tagged-NTN on the surface of target cells may be detected using immunofluorescent techniques in which a molecule which reacts with the tag, preferably an antibody, directly or indirectly produces fluorescent light. The fluorescence may either be observed under a microscope or used to segregate tagged-NTN-bearing cells by fluorescence activated cell sorting techniques. The present invention also provides for methods for detecting other forms of tags, such as chromogenic tags and catalytic tags. An anti-NTNR $\alpha$  antibody can also be used as a probe. The detection methods for any particular tag will depend on the conditions necessary for producing a signal from the tag, but should be readily discernible by one skilled in the art.

NTNR $\alpha$  variants are useful as standards or controls in assays for the NTNR $\alpha$  for example ELISA, RIA, or RRA, provided that they are recognized by the analytical system employed, *e.g.*, an anti-NTNR $\alpha$  antibody.

Polyclonal antibodies are generally raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. In that the preferred epitope is in the ECD of the NTNR $\alpha$ , it is desirable to use NTNR $\alpha$  ECD or a molecule comprising the ECD (*e.g.*, NTNR $\alpha$  immunoadhesin) as the antigen for generation of polyclonal and monoclonal antibodies. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl<sub>2</sub>, or R<sup>1</sup>N=C=NR, where R and R<sup>1</sup> are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining 1 mg or 1  $\mu$ g of the peptide or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the

conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

5 Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al.*, *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (Cabilly *et al.*,  
10 *supra*).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol,  
15 to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase  
20 (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MORC-21  
25 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

30 Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard  
35 analysis of Munson *et al.*, *Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *supra*). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The ability of the MAbs to block binding of NTN to its receptor can be evaluated by ELISA and  
5 bioassay utilizing available reagents (rhNTNr-IgG; a stable transfected CHO cell line expressing NTN $\alpha$ ). Neutralizing activities can also be evaluated by neuronal survival assay(s).

NTNR-specific MAbs can be developed as discussed above using for example, the receptor immunoadhesin and transfected cell line) to initiate new immunization protocols to generate NTNR-specific MAbs for use as potential agonists or antagonists, as well as for immunohistochemistry,  
10 immunocytochemistry, and assay development. The MAbs generated from fusion of the immunized animals can be screened for agonist and antagonist activities by bioassay (e.g., neuron survival assays, signal transduction/phosphorylation, kidney cell survival assays) as well as by ELISA and FACS (functional blocking of NTN-NTN $\alpha$  binding). Suitable techniques are provided in, for example, Lucas *et al.*, J. Immunol. 145:1415-1422 (1990); Hoogenraad *et al.* J. Immunol. Methods 6:317-320 (1983); Moks *et al.*, Eur. J.  
15 Biochem. 85:1205-1210 (1986); Laemmli, Nature (London) 227:680-685 (1970); and, Towbin *et al.*, Proc Natl Acad Sci USA 76:4350-4354 (1979).

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such  
20 DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra *et al.*, *Curr. Opinion in Immunol.*, 5:256-262 (1993) and Plückthun, *Immunol. Revs.*, 130:151-  
25 188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al.*, *Nature*, 348:552-554 (1990). Clackson *et al.*, *Nature*, 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the  
30 production of high affinity (nM range) human antibodies by chain shuffling (Mark *et al.*, *BioTechnology*, 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.*, *Nuc. Acids. Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

35 The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (Cabilly *et al.*, *supra*; Morrison, *et al.*, *Proc. Nat. Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

5 Chimeric or hybrid antibodies also may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide-exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

10 Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.*, *Nature*, 321:522-525 (1986); Riechmann *et al.*, *Nature*, 332:323-327 (1988); Verhoeyen *et al.*, *Science*, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the  
15 corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (Cabilly *et al.*, *supra*), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

20 The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims *et al.*, *J. Immunol.*, 151:2296 (1993); Chothia *et al.*, *J. Mol. Biol.*, 196:901 (1987)). Another method uses a particular framework derived from the consensus  
25 sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta *et al.*, *J. Immunol.*, 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and  
30 other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of  
35 selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequences so that the desired antibody characteristic,

such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region ( $J_H$ ) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al.*, *Nature*, 362:255-258 (1993); Bruggermann *et al.*, *Year in Immuno.*, 7:33 (1993). Human antibodies can also be produced in phage-display libraries (Hoogenboom *et al.*, *J. Mol. Biol.*, 227:381 (1991); Marks *et al.*, *J. Mol. Biol.*, 222:581 (1991)).

Bispecific antibodies (BsAbs) are antibodies that have binding specificities for at least two different antigens. BsAbs can be used as tumor targeting or imaging agents and can be used to target enzymes or toxins to a cell possessing the NTN $\alpha$ . Such antibodies can be derived from full length antibodies or antibody fragments (e.g. F(ab')<sub>2</sub> bispecific antibodies). In accordance with the present invention, the BsAb may possess one arm which binds the NTN $\alpha$  and another arm which binds to a cytokine or another cytokine receptor (or a subunit thereof) such as the receptors for TPO, EPO, G-CSF, IL-4, IL-7, GH, PRL; the  $\alpha$  or  $\beta$  subunits of the IL-3, GM-CSF, IL-5, IL-6, LIF, OSM and CNTF receptors; or the  $\alpha$ ,  $\beta$  or  $\gamma$  subunits of the IL-2 receptor complex. For example, the BsAb may bind both NTN $\alpha$  and gp130.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein *et al.*, *Nature*, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker *et al.*, *EMBO J.*, 10:3655-3659 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression

vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published March 3, 1994. For further details of generating bispecific antibodies see, for example, Suresh *et al.*, *Methods in Enzymology*, 121:210 (1986).

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. The following techniques can also be used for the production of bivalent antibody fragments which are not necessarily bispecific. According to these techniques, Fab'-SH fragments can be recovered from *E. coli*, which can be chemically coupled to form bivalent antibodies. Shalaby *et al.*, *J. Exp. Med.*, 175:217-225 (1992) describe the production of a fully humanized BsAb F(ab')<sub>2</sub> molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the BsAb. The BsAb thus formed was able to bind to cells overexpressing the HER2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets. See also Rodriguez *et al.*, *Int. J. Cancers*, (Suppl.) 7:45-50 (1992).

Various techniques for making and isolating bivalent antibody fragments directly from recombinant cell culture have also been described. For example, bivalent heterodimers have been produced using leucine zippers. Kostelny *et al.*, *J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. The "diabody" technology described by Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making BsAb fragments. The fragments comprise a heavy-chain variable domain (V<sub>H</sub>) connected to a light-chain variable domain (V<sub>L</sub>) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V<sub>H</sub> and V<sub>L</sub> domains of one fragment are forced to pair with the complementary V<sub>L</sub> and V<sub>H</sub> domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making BsAb fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al.*, *J. Immunol.*, 152:5368 (1994).

The NTN $\alpha$  agonists (including NTN) and agonist NTN $\alpha$  antibodies of the present invention can be used to enhance splenic hematopoiesis, allowing some repopulation of blood cell lineages in patients having



undergone chemo- or radiation therapy and transplantation. Generally, the antibodies will act to enhance proliferation and/or differentiation (but especially proliferation) of hematopoietic cells in the spleen. Without being bound by theory, NTNR $\alpha$  agonists may act directly as a growth, survival or differentiation factor for hematopoietic cells in the spleen and/or may indirectly act on the splenic stromal environment (possibly neurons involved in the splenic innervation) to produce another factor that is responsible for the maintenance of hematopoietic lineages. In any event, as taught herein NTNR $\alpha$  agonist, including NTN, have therapeutic benefit in facilitating the splenic engraftment of bone marrow transplants following irradiation or chemotherapy or for stimulating extramedullary hematopoiesis in the spleen (which is normal in rodents, but not normally seen in man) in those conditions where there is an increased demand for blood cell production due to anemia (red blood cells), chronic infection (neutrophils), bone marrow failure (all lineages), and immune deficiency (lymphocytes). The agonists may similarly be useful for treating diseases characterized by a decrease in blood cells. Examples of these diseases include: anemia (including macrocytic and aplastic anemia); thrombocytopenia; hypoplasia; immune (autoimmune) thrombocytopenic purpura (ITP); and HIV induced ITP. Also, the agonists may be used to treat a patient having suffered a hemorrhage.

Therapeutic applications for NTNR $\alpha$  neutralizing antibodies include the treatment of metabolic disorders and cell tumors at sites of NTNR $\alpha$  expression, especially those tumors characterized by overexpression of NTNR $\alpha$ .

For therapeutic applications, the NTNR $\alpha$  antibodies of the invention are administered to a mammal, preferably a human, in a physiologically acceptable dosage form, including those that may be administered to a human intravenously as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intra-cerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. The NTNR $\alpha$  antibodies also are suitably administered by intratumoral, peritumoral, intralesional, or perilesional routes or to the lymph, to exert local as well as systemic therapeutic effects.

Such dosage forms encompass physiologically acceptable carriers that are inherently non-toxic and non-therapeutic. Examples of such carriers include ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts, or electrolytes such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, and PEG. Carriers for topical or gel-based forms of NTNR $\alpha$  antibodies include polysaccharides such as sodium carboxymethylcellulose or methylcellulose, polyvinylpyrrolidone, polyacrylates, polyoxyethylene-polyoxypropylene-block polymers, PEG, and wood wax alcohols. For all administrations, conventional depot forms are suitably used. Such forms include, for example, microcapsules, nano-capsules, liposomes, plasters, inhalation forms, nose sprays, sublingual tablets, and sustained-release preparations. The NTNR $\alpha$  antibody will typically be formulated in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml.

Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the NTNR $\alpha$  antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, *supra* and Langer, *supra*, or

poly(vinylalcohol), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and  $\gamma$  ethyl-L-glutamate (Sidman *et al.*, *supra*), non-degradable ethylene-vinyl acetate (Langer *et al.*, *supra*), degradable lactic acid-glycolic acid copolymers such as the Lupron Depot<sup>TM</sup> (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers  
5 such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated NTN $\alpha$  antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is  
10 discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release NTN $\alpha$  antibody compositions also include liposomally entrapped antibodies. Liposomes containing the NTN $\alpha$  antibodies are prepared by methods known in the art, such as described in  
15 Epstein *et al.*, *Proc. Natl. Acad. Sci. USA*, 82:3688 (1985); Hwang *et al.*, *Proc. Natl. Acad. Sci. USA*, 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Ordinarily, the liposomes are the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol.% cholesterol, the selected proportion being adjusted for the optimal NTN $\alpha$  antibody therapy. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

20 For the prevention or treatment of disease, the appropriate dosage of NTN $\alpha$  antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibodies are administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the NTN $\alpha$  antibody, and the discretion of the attending physician. The NTN $\alpha$  antibody is suitably administered to the patient at one time or over a series of treatments.

25 Depending on the type and severity of the disease, about 1  $\mu$ g/kg to 15 mg/kg of NTN $\alpha$  antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1  $\mu$ g/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease  
30 symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Animal model are available to assess effects of the compounds and method of the invention. For example, to assess the effects of treating damaged kidneys with compositions that affect growth (Toback, 1977; Toback *et al.* 1977), an intravenous injection of 1.0 to 1.1 mg of mercury per kg of body weight as  
35 HgCl<sub>2</sub> is given to rats to induce a reversible syndrome of acute nonoliguric acute renal failure. After one day, there are marked increases in serum urea nitrogen concentration (SUN), urinary excretion of sodium and protein, and necrosis of proximal tubular cells. By day two, increases in phospholipid, DNA and RNA synthesis, and mitotic index indicate that cellular regeneration is underway. By day three, the SUN reaches a maximum, and squamoid epithelial cells appear on the tubular basement membrane. At day five, the SUN

returns to normal, the maximal rate of phospholipid synthesis is reached, and the tubules are repopulated with more mature cells. The effects of infusion of a composition of autocrine growth factors on renal structure is compared with untreated rats and animals infused with vehicle alone during the course of the mercuric chloride-induced acute tubular necrosis syndrome discussed above.

- 5       The NTNR $\alpha$  antibodies of the invention are also useful as affinity purification agents. In this process, the antibodies against NTNR $\alpha$  are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the NTNR $\alpha$  to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the NTNR $\alpha$ , which is bound to the immobilized antibody.
- 10       Finally, the support is washed with another suitable solvent, such as glycine buffer, pH 5.0, that will release the NTNR $\alpha$  from the antibody.

NTNR $\alpha$  antibodies may also be useful in diagnostic assays for NTNR $\alpha$ , *e.g.*, detecting its expression in specific cells, tissues, or serum. For diagnostic applications, antibodies typically will be labeled with a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or

15       indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ , or  $^{125}\text{I}$ ; a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; radioactive isotopic labels, such as, *e.g.*,  $^{125}\text{I}$ ,  $^{32}\text{P}$ ,  $^{14}\text{C}$ , or  $^3\text{H}$ ; or an enzyme, such as alkaline phosphatase, beta-galactosidase, or horseradish peroxidase.

Any method known in the art for separately conjugating the polypeptide variant to the detectable

20       moiety may be employed, including those methods described by Hunter *et al.*, *Nature*, 144:945 (1962); David *et al.*, *Biochemistry*, 13:1014 (1974); Pain *et al.*, *J. Immunol. Meth.*, 40:219 (1981); and Nygren, *J. Histochem. and Cytochem.*, 30:407 (1982).

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola,

25       *Monoclonal Antibodies: A Manual of Techniques*, pp.147-158 (CRC Press, Inc., 1987).

Competitive binding assays rely on the ability of a labeled standard to compete with the test sample analyte for binding with a limited amount of antibody. The amount of NTNR $\alpha$  in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies generally are insolubilized before or after the

30       competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds

35       to the analyte, thus forming an insoluble three-part complex. See, *e.g.*, US Pat No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

The following Examples of specific embodiments for carrying out the present invention are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

The disclosures of all publications, patents and patent applications cited herein are hereby incorporated by reference in their entirety.

## EXAMPLES

### Example 1

#### Cloning of Human NTNR $\alpha$

Eight partial human cDNAs (Genbank accession numbers: R02249 (SEQ ID NO.: 7), H12981 (SEQ ID NO.: 8), W73681 (SEQ ID NO.: 9), W73633 (SEQ ID NO.: 10), H05619 (SEQ ID NO.: 11), R02135 (SEQ ID NO.: 12), T03342 (SEQ ID NO.: 13), and HSC1KA111 (SEQ ID NO.: 14)) were identified as having similarity to the GDNF receptor  $\alpha$  component (Jing *et al. Cell* 85:1113-1124 (1996); Treanor *et al. Nature* 382:80-83 (1996)), but were not identical to GDNFR sequences. A DNA sequence, determined by aligning these expressed-sequence-tag ("EST") cDNA sequences, was extended using 5' and 3' Marathon RACE reactions (Clontech Inc.) on human spleen mRNA, using conditions supplied by the manufacturer, to obtain an initial set of human cDNA clones. Additional cDNA clones were identified by screening a human fetal brain cDNA library (Stratagene) using standard protocols. Lambda cDNA libraries were plated using standard protocols and a coding region probe, obtained by PCR amplification of the NTNR $\alpha$  gene using the 3' and 5' RACE information, was hybridized to the library. From an alignment of the cloned human cDNA sequences, a full length cDNA sequence was obtained (SEQ ID NO: 1), which was referred to as the human Neurturin receptor  $\alpha$  ("hNTNR $\alpha$ ") cDNA sequence. This sequence contained a open reading frame sequence (SEQ ID NO: 2) that encoded a single 464 amino acid protein sequence (SEQ ID NO: 3), which was designated human Neurturin receptor  $\alpha$  ("hNTNR $\alpha$ ").

Human NTNR $\alpha$  ("hNTNR $\alpha$ ") displays an overall 47% similarity at the amino acid level to both hGDNFR $\alpha$  and rGDNFR $\alpha$ .

hNTNR $\alpha$ , like hGDNFR $\alpha$ , is an extracellular protein that is attached to the outer cell membrane via a glycosyl-phosphatidyl inositol ("GPI") modification. It has an amino terminal signal peptide for secretion, 3 potential glycosylation sites, and a stretch of 17 carboxy terminal hydrophobic amino acids preceded by a group of 3 small amino acids (Gly, Ser, Asn) defining a cleavage/binding site for GPI linkage (Fig. 4; Micanovic *et al., Proc. Natl. Acad. Sci. USA*, 87:157-161 (1990); Moran *et al., J. Biol. Chem.*, 266:1250-1257 (1991)). The position of their 30 cysteine residues are completely conserved between NTNR $\alpha$  and GDNFR $\alpha$  (Fig. 5). The extracellular domain ("ECD") is flanked by the signal peptide and the GPI-attachment site.

### Example 2

#### Cloning of Rat NTNR $\alpha$ ("rNTNR $\alpha$ ")

Rat NTNR $\alpha$  ("rNTNR $\alpha$ ") was cloned by screening a rat brain cDNA library (Clontech) using standard protocols. A full-length human NTNR $\alpha$  probe was hybridized to the rat cDN library at moderate stringency (e.g., 30% formamide at 42°C, wash in 0.1 x SSC at 55°C). The cDNA, having (SEQ ID NO: 4) and containing the complete open reading frame (SEQ ID NO: 5), was designated as rNTNR $\alpha$  cDNA. The open reading frame sequence encoded a single 464 amino acid protein sequence (SEQ ID NO: 6), which was designated rat Neurturin receptor  $\alpha$  ("rNTNR $\alpha$ ").

Rat NTNR $\alpha$  and human NTNR $\alpha$  display an overall 94% similarity at the amino acid level. At the DNA level, a 79% identity was observed. Rat NTNR $\alpha$  is 46% identical to both rat GDNFR $\alpha$  and human GDNFR $\alpha$  at the protein level. Rat NTNR $\alpha$  is 53% identical to rat GDNFR $\alpha$  at the DNA level.

5 rNTNR $\alpha$ , like rGDNFR $\alpha$ , hGDNFR $\alpha$ , and hNTNR $\alpha$ , is an extracellular protein that is attached to the outer cell membrane via a glycosyl-phosphatidyl inositol ("GPI") modification. It has an amino terminal signal peptide for secretion, 3 potential glycosylation sites, and a stretch of 17 carboxy terminal hydrophobic amino acids preceded by a group of 3 small amino acids (Gly, Ser, Asn) defining a cleavage/binding site for GPI linkage (Fig. 4). Surprisingly, the position of the 30 cysteine residues, which are conserved between human and rat GDNFR $\alpha$ , are conserved between human and rat NTNR $\alpha$  (Fig. 5). These cysteine residues are completely conserved between NTNR $\alpha$  and GDNFR $\alpha$  (Fig. 5). The extracellular domain ("ECD") is flanked by the signal peptide and the GPI-attachment site.

### Example 3

#### Vectors for Expression of Membrane-Bound and Soluble NTNR $\alpha$

For mammalian protein expression the complete open reading frame of human NTNR $\alpha$  was amplified using PCR and cloned into a CMV based expression vector pRK5 or pRK7. The plasmid was designated pRK-hNTNR $\alpha$  for the human NTNR.

To make soluble forms rat and human NTNR $\alpha$ , both rat and human NTNR $\alpha$ -IgG expression construct were made by cloning the first 432 amino acids of each receptor (which lacks a GPI linkage site) in front of the human Fc sequence. For example, the NTNR $\alpha$ -IgG expression construct was made by cloning the first 432 amino acids of the receptor (which lacks a GPI linkage site) in front of the human Fc (IfF2a) sequence. Plasmids were designated pRK-hNTNR $\alpha$ -IgG for hNTNR $\alpha$  fusion and pRK-rNTNR $\alpha$ -IgG for rat NTNR $\alpha$  fusion. The human gene fusion coding nucleic acid sequence is SEQ ID NO: 15, which encodes the human fusion protein SEQ ID NO: 16. Suitable locations for attachment of structure to the ECD are within, or preferably C-terminal to, the CFTELTTNIIPG sequence of NTNR $\alpha$  ECD. The rat gene fusion coding nucleic acid sequence is SEQ ID NO: 17, which encodes the rat fusion protein SEQ ID NO: 18.

### Example 4

#### Tissue distribution of NTNR $\alpha$ .

The tissue distribution of the NTNR $\alpha$  mRNA was examined using *in situ* hybridization analysis. Its distribution was compared to that of GDNFR $\alpha$ .

For *in situ* hybridization, E15.5 rat embryos were immersion-fixed overnight at 4°C in 4% paraformaldehyde, then cryoprotected overnight in 15% sucrose. Adult rat brains and spinal cords were frozen fresh. All tissues were sectioned at 16  $\mu$ m, and processed for *in situ* hybridization using <sup>33</sup>P-UTP labelled RNA probes as described (Davis *et al. Science* 259:1736-1739 (1993)). Sense and antisense probes were derived from the N-terminal region of GDNFR $\alpha$  using T7 polymerase. NTNR $\alpha$  RNA was detected with a probe, derived from hNTNR $\alpha$ , designated hNTNR $\alpha$ .T7insitu probe (SEQ ID NO: 19).

In the embryonic and adult rat nervous system, mRNA for NTNR $\alpha$  was found in the ventral midbrain, where dopaminergic neurons are located, in parts of the ventral spinal cord where motoneurons are located, and in the dorsal root ganglia (DRG) where sensory neurons reside. In addition, high levels of NTNR $\alpha$  transcripts were found in tissues such as the embryonic gut, bladder, cardiac conduction system and

diaphragm. In the adult rat brain NTNR $\alpha$  was found mainly in the substantia nigra, cortex and olfactory bulb as well as in the dorsal horn of the spinal cord. Although NTNR $\alpha$  was occasionally found in tissues that express GDNFR $\alpha$ , the two transcripts were most often present adjacent to each other. For example, in the limb, GDNFR $\alpha$  is expressed in muscle cells, whereas NTNR $\alpha$  is found in the brachial plexus nerve which  
5 enervates the muscle. Likewise, in the embryonic bladder, NTNR $\alpha$  is expressed in the muscle layer, while GDNFR $\alpha$  is expressed in the underlying epithelia. Finally, in the gut NTNR $\alpha$  is expressed in the mucosal epithelium, while GDNFR $\alpha$  is expressed only in the adjacent smooth muscles. This pattern of expression is consistent with the notion that NTNR $\alpha$  mediates signals inside, as well as outside, the nervous system, and suggests distinct, complementary biological roles for NTNR $\alpha$  and GDNFR $\alpha$ .

#### 10 Example 5

##### NTNR $\alpha$ Specifically Binds NTN

Equilibrium binding experiments were performed to determine the binding of NTNR $\alpha$  to NTN. Conditioned media from 293 cells transiently transfected with either the pRK-hNTNR $\alpha$ -IgG or pRK-rNTNR $\alpha$ -IgG constructs provided soluble receptor. The conditioned media was incubated with approximately 5 pM  
15  $^{125}$ I human Neurturin, mouse Neurturin, or rGDNF along with different concentrations of the appropriate cold ligand in PBS containing 2 mg/ml BSA (Sigma) and 0.05% Brij 96 (Fluka) for 4 hours at room temperature. Ligand is in excess over the receptor. Receptor/ligand complexes were then incubated with protein A Sepharose CL-4B (Pharmacia) for an additional 1 hour at room temperature. After washing with PBS containing 0.2 mg/ml BSA, specific perceptible counts were measured. The IGOR program was used to  
20 determine K<sub>d</sub>. It was found that both human and mouse  $^{125}$ I-NTN can bind to recombinant soluble human NTNR $\alpha$  (data not shown) or soluble rNTNR $\alpha$  protein, specifically and reversibly with an approximate K<sub>d</sub> of 10 pM (inset to Fig. 6C). In contrast neither human NTN (data not shown) nor mouse NTN were able to displace  $^{125}$ I-rGDNF from rGDNFR $\alpha$  (Fig. 6B), and no high affinity binding of human or mouse  $^{125}$ I-NTN to rGDNFR $\alpha$  was detected (Fig. 6A). Accordingly, NTN specifically binds NTNR $\alpha$ , interacting with high  
25 affinity with NTNR $\alpha$  but not with rGDNFR $\alpha$ . To further confirm that NTNR $\alpha$  is a specific receptor for NTN, competition binding experiments were performed using  $^{125}$ I-rGDNF. Displaceable high affinity binding of  $^{125}$ I-rGDNF to recombinant soluble rGDNFR $\alpha$  was readily observed (K<sub>d</sub> of 3 pM) (Fig. 6B inset); however, binding of  $^{125}$ I-rGDNF (iodinated either on tyrosine by the Bolton Hunter method or on lysine using lactoperoxidase) to either human or rat NTNR $\alpha$  was not detected. GDNF interacts at high affinity with  
30 GDNFR $\alpha$  but not with NTNR $\alpha$ . And, NTN specifically binds NTNR $\alpha$ . While no high affinity interaction between NTN and GDNFR $\alpha$  was detected, a low affinity interaction (K<sub>d</sub> > 1nM) was observed when higher concentrations of unlabeled NTN (10 nM) displaced labelled rGDNF from GDNFR $\alpha$ .

Cell based equilibrium binding analysis, performed as described (Treanor *et al. Nature* 382:80-83 (1996)), confirmed the specificity of GDNFR $\alpha$  and NTNR $\alpha$  for GDNF and Neurturin, respectively. 293 cells,  
35 which were transiently transfected with either the full length NTNR $\alpha$  expression construct (or GDNFR $\alpha$ ) or an irrelevant (control) construct, provided membrane-bound receptor. The observed specific associations between GDNF and GDNFR $\alpha$  and between NTN and NTNR $\alpha$  using competition binding to cells that express

The ligand-binding effect of phosphoinositide-specific phospholipase C ("PIPLC") treatment of the membrane bound receptor was determined. PIPLC is an enzyme that specifically cleaves GPI linkage (Koke *et al. Prot. Express. Purification* 2:51-58 (1991); Shukla, *Life Sci.*, 10:1323-1335 (1982); Rhee *et al., Science*, 244:546-550 (1989)). 293 cells, which were transiently transfected with either the full length NTN $\alpha$  expression construct or an irrelevant (control) construct, were incubated with ~20,000 cpm  $^{125}$ I human NTN in the presence of the indicated amounts of PIPLC for 90 minutes at room temperature (Fig. 7A). The cells were washed with ice-cold PBS containing 0.2 mg/ml BSA, after which cell associated  $^{125}$ I was measured. Consistent with the prediction that NTN $\alpha$  is anchored to the cell surface by a GPI linkage, the binding of  $^{125}$ I-NTN to cells expressing NTN $\alpha$  was significantly reduced following treatment with (Fig. 7A). Accordingly, NTN $\alpha$  is a specific high affinity GPI-linked receptor for NTN.

#### Example 6

##### NTN $\alpha$ Mediates Biological Response to NTN

To confirm that NTN $\alpha$  mediates the biological response to NTN, the effect of NTN on the survival of cells that express NTN $\alpha$  was determined. For survival assays E14 rat motoneurons were isolated, plated, and grown in triplicate wells as described. After addition of the indicated growth factors, the number of surviving neurons was determined 72 hours later (Figure 7B). It was observed that NTN can prevent the death of primary motoneurons (Fig. 7B) that express NTN $\alpha$  (as determined by in situ hybridization). Moreover, in agreement with the finding that NTN and GDNF utilize distinct receptors, quantitative differences in the survival response of motoneurons to the two factors was detected: GDNF at saturating concentrations promoted the survival of 100% of the BDNF-responsive motoneurons; NTN, whose receptor is sparsely distributed in the embryonic ventral horn where motoneurons reside, prevented the death of only 50% of these cells (Fig. 7B). It was also observed that NTN can prevent the death of primary embryonic dopaminergic neurons that express NTN $\alpha$ .

To further confirm that NTN $\alpha$  is a required mediator of the NTN signal, embryonic motoneurons were treated with PIPLC and their survival in the presence of NTN or BDNF was monitored in culture. E14 rat motoneurons were isolated, plated, and grown in triplicate wells as described. PIPLC (2-4ug/ml) was added to the indicated samples 1-2 h prior to, as well as 12h and 24h following, addition of the indicated growth factors, and the number of surviving neurons was determined 72h later (Figure 7C). The number of embryonic rat spinal motoneurons that remained alive at saturating concentrations of NTN was reduced by 70-90% following PIPLC treatment, whereas no decrease in the response of PIPLC-treated neurons to brain-derived neurotrophic factor (BDNF) was noted. Moreover, when NTN was added to these motoneurons in combination with soluble NTN $\alpha$  (IgG fusion), the response to NTN was restored (Fig. 7C). Accordingly, NTN $\alpha$  appears to be an essential component of the NTN signaling cascade and has the properties expected of the ligand-binding subunit of a functional NTN receptor. In addition, the soluble NTN $\alpha$  receptor can impart a NTN-responsiveness to cells that lack cell surface NTN $\alpha$ , but express the complementing Ret transmembrane protein.

## Example 7

NTNR $\alpha$  Can Act Via Ret

Since NTNR $\alpha$ , like GDNFR $\alpha$ , lacks a cytoplasmic domain and appears to be anchored to the outer surface of the cell via GPI, transmission of the NTN signals to the cell interior must involve additional proteins. As the tyrosine kinase receptor Ret, which by itself does not bind GDNF or NTN with a high affinity (Jing *et al. Cell* 85:1113-1124 (1996)); Treanor *et al. Nature* 382:80-83 (1996); data not shown), appears to be a signaling component of the GDNF receptor, Ret transduction of the NTN response following binding of NTN to NTNR $\alpha$  was determined. To assay for tyrosine phosphorylation, cells were incubated for 1h at 37°C with or without PIPLC, and then exposed to various concentrations of NTN. Cells were then removed from the plates with 2mM EDTA in PBS and lysed with ice-cold buffer (10 mM sodium phosphate (pH 7.0), 100 mM NaCl, 1% NP40, 5 mM EDTA, 100 mM sodium vanadate, 2 mM PMSF, and 0.2 units of aprotinin), and used for immunoprecipitation with antisera raised against the 19 amino acid carboxyl terminus of Ret, followed by binding to protein A sepharose. The immunoprecipitated proteins were released by boiling in SDS sample buffer, separated on an 8% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and reacted with anti-phosphotyrosine antibody (Upstate Biotechnology, Inc.); detection was with an ECL Western blotting detection system (Amersham Life Science). The human neuroblastoma cell line, TGW-1, which expresses endogenous *c-ret* (Ikeda *et al. Oncogene* 5:1291 (1990); Takahashi *et al. Oncogene* 6:297 (1991)), was exposed to NTN for 5 minutes, and the level of Ret tyrosine phosphorylation was determined. NTN clearly induced phosphorylation of Ret (Fig. 7D), as well as of the receptor tyrosine kinase responsive, cytoplasmic kinase ERK (i.e., MAPK) in this cell line (Fig. 7E), but not in 4 other neuroblastoma lines that were examined (data not shown). Furthermore, consistent with the hypothesis that NTNR $\alpha$  is an essential mediator between NTN and Ret, NTN failed to induce significant tyrosine phosphorylation on Ret in cells that were treated with PIPLC (Fig. 7F). A similar result was obtained with GDNF. Tyrosine-phosphorylated RET protein was readily detected in PIPLC-treated TGW-1 cell when NTN was added together with a soluble NTNR $\alpha$ .

Since these findings herein suggested that Ret participates in the transmission of the NTN signal, it was determined whether Ret is part of a putative NTN receptor complex. To examine the formation of protein complexes upon exposure to NTN, co-immunoprecipitation experiments were done NTNR $\alpha$ -expressing TGW-1 cells exposed to 500 ng/ml of NTN. After exposure, cells were lysed with a mild detergent brij 96 (Sigma) (Davis *et al.* 1993). For mammalian protein expression the complete open reading frame was amplified using PCR and cloned into a CMV based expression vector. For co-precipitation experiments, an epitope tag was inserted between the signal peptide and the mature coding sequence of NTNR $\alpha$ . When protein complexes were immunoprecipitated with a polyclonal antibody to Ret and then analyzed on a western blot using a polyclonal antibody to NTN, NTN was readily co-immunoprecipitated by Ret antibodies, which is consistent with the notion that NTN and Ret physically interact on the cell surface. To confirm that NTNR $\alpha$  is part of the NTN/Ret protein complex, human embryonic kidney 293 cells were transiently transfected with expression vectors, Ret alone or with a combination of expression vectors for *c-ret* and an epitope tagged NTNR $\alpha$ , exposed to NTN, and lysed with a mild detergent brij 96 (Sigma) (Davis *et al.* 1993). Putative immune complexes were immunoprecipitated with a polyclonal antibody against Ret, transferred onto a nitrocellulose



filter, and analyzed with a polyclonal antibody against the epitope tagged NTN $\alpha$ . In agreement with the idea that NTN $\alpha$  and Ret can be found in a protein complex, NTN $\alpha$ , in the presence, but not in the absence of NTN was readily co-immunoprecipitated by Ret antibodies. These findings demonstrated that NTN, NTN $\alpha$  and Ret can form a complex on the cell surface, that Ret and NTN $\alpha$  are components of a functional NTN receptor, and that NTN $\alpha$  is an intermediary in the interaction between NTN and Ret.

To ascertain the potential role of NTN $\alpha$  in the survival responses of developing neurons to neurotrophic factors and compare its function with that of GDNF $\alpha$ , microinjection was used to introduce expression plasmids encoding NTN $\alpha$  and GDNF $\alpha$  into cultured neurons that normally do not survive in response to either GDNF or neurturin. Although neurturin promotes the survival of late fetal rat sympathetic neurons (Kotzbauer, 1996), from a survey of several different populations of neurons, it was found herein that sympathetic neurons of the superior cervical sympathetic ganglion (SCG) of postnatal day 4 (P4) mice are not supported by either neurturin or GDNF in culture. Because these neurons are also relatively easy to microinject and die rapidly in defined medium lacking neurotrophic factors, they are very useful for examining the involvement of NTN $\alpha$  in neuronal survival and neurotrophic factor responsiveness. Ectopic expression of either NTN $\alpha$  or GDNF $\alpha$  alone in SCG neurons had a negligible effect on the survival response of these neurons to either GDNF or neurturin (less than 5% survive in medium containing these factors following injection with either NTN $\alpha$  or GDNF $\alpha$  expression plasmids). This suggests that neither GDNF $\alpha$  nor NTN $\alpha$  alone are capable of mediating survival responses to GDNF and neurturin. This is consistent with the idea that GPI-linked receptors cannot mediate responses to their ligands without the appropriate transmembrane signaling proteins like Ret in the case of GDNF $\alpha$  (Treanor et al., 1996; Jing et al., 1996) and gp130 and LIFR $\beta$  in the case of CNTF $\alpha$  (Davis et al., 1993).

Ret was reported as an essential signaling component of the GDNF receptor complex (Treanor et al., 1996; Jing et al., 1996; Trupp et al., 1996; Durbec et al., 1996; Vega, 1996). To see if neurons expressing both of these receptor components would exhibit specific survival responses to GDNF and neurturin, neurons were co-injected with expression plasmids for Ret and NTN $\alpha$  or GDNF $\alpha$ . Ectopic expression of Ret alone had only a small effect on the number of SCG neurons surviving in the presence of neurturin or GDNF (between 10 and 15%). However, neurons co-expressing NTN $\alpha$  plus Ret had a substantially enhanced survival response to neurturin that was significantly greater than that of neurons expressing Ret alone ( $p = 0.003$ , t-test,  $n = 6$ ). Likewise, neurons co-expressing GDNF $\alpha$  plus Ret had substantially enhanced survival response to GDNF that was also significantly greater than that of neurons expressing Ret alone ( $p = 0.0002$ , t-test,  $n = 9$ ). In contrast, neurons co-expressing GDNF $\alpha$  plus Ret showed no enhanced survival response to neurturin; there were no more Ret/GDNF $\alpha$ -expressing neurons surviving with neurturin than Ret-expressing neurons. Likewise, the number of Ret/NTN $\alpha$ -expressing neurons surviving with GDNF was not significantly different from the number of Ret-expressing neurons surviving with this factor ( $p = 0.2$ , t-test,  $n = 9$ ). These data confirm the results reported above.

#### Example 8

##### NTN Is a Neuron Survival Factor In Vivo

To determine whether NTN can act to promote survival of midbrain DA neurons, cultures of E14 rat ventral midbrain, enriched for DA neurons, were investigated. This culture system revealed that, like GDNF,

NTN can exert potent actions on the survival of tyrosine hydroxylase-expressing cells of the developing midbrain. The potency and efficacy of NTN was similar to that of GDNF, with NTN displaying a trend towards promoting survival of greater numbers of cells than seen with maximally-effective doses of GDNF.

The ability of NTN to promote survival *in vitro* of embryonic rat midbrain DA neurons suggests that NTN can be effective in promoting survival of DA neurons in the intact adult brain. As indicated herein, the tyrosine kinase Ret is a critical component of both GDNF and NTN receptor complexes and is expressed in adult rat midbrain DA neurons. To determine whether NTNR $\alpha$  is expressed in adult nigral DA neurons, *in situ* hybridization was employed. While sections of the adult rat ventral midbrain displayed strong signal for GDNFR $\alpha$ , which was largely confined to the pars compacta of the substantia nigra and the ventral tegmental area, a more modest and diffuse signal for signal for NTNR $\alpha$  was observed in the ventral midbrain. In sections stained for tyrosine hydroxylase, the majority of TH+ nigral cells displayed weak or equivocal signal for NTNR $\alpha$ , while intense hybridization was observed for GDNFR $\alpha$  in association with TH+ cells of the substantia nigra. Strong signal for NTNR $\alpha$  was, however, observed in regions immediately adjacent to midbrain DA neurons, including cells bordering the dorsolateral aspect of the pars compacta of the substantia nigra, the medial and lateral nuclei of the accessory optic tract, and interpeduncular nuclei. Taken with the ability of soluble NTNR $\alpha$  expression in and near adult nigral DA neurons, these results suggest that NTN is acting on adult nigral neurons.

It was determined herein that intranigral injection of NTN can promote the survival and TH expression of DA neurons following striatal 6-OHDA administration, and that the potency and efficacy of NTN is similar to that of GDNF. In order to independently assess viability and phenotypic expression of nigral DA neurons, cells were counted using both a retrograde fluorescent tracer, flurogold, and by immunocytochemistry for tyrosine hydroxylase. A single intranigral injection of 1 or 10 ug of NTN administered one week following 6-OHDA administration lead to nearly 3 fold higher cell viability (as assessed by the retrograde tracer flurogold) at one month post-lesion than seen in vehicle-treated animals. Examination of the number of cells expressing tyrosine hydroxylase revealed a significant increase in protection in rats treated with 10, but not 1 microgram of NTN. The effects of single injections of NTN on cell survival and TH expression were indistinguishable from that seen with comparable doses of GDNF. Both NTN and GDNF can promote the survival of nigral DA neurons following neurotoxic or traumatic injury.

As shown herein, NTN is expressed in the developing and adult nigrostriatal system and can exert potent influences on the survival and phenotypic expression of nigral dopaminergic neurons. The actions of NTN to protect DA neurons and promote TH expression following 6-OHDA administration indicate that this factor can be a useful agent in the treatment of Parkinson's disease.

#### Example 9

##### NTNR $\alpha$ and GDNFR $\alpha$ Extracellular Domains Act as Receptor Agonists

The effects of GDNFR $\alpha$  and NTNR $\alpha$  extracellular domains as receptor agonists were determined by observing survival of ventral mesencephalic, embryonic rat, dopaminergic neurons treated with exogenously added extracellular domain of either receptor. Cultures enriched for dopaminergic neurons of the ventral mesencephalon were dissected from E14 rats, in which Day 0 was the day of the first appearance of the vaginal plug. The tissue was treated with enzyme, triturated to a single cell suspension, and plated as previously

described (Poulsen *et al.*, *Neuron* 13(5):1245-52 (1994)) with a few exceptions. Cells were plated on glass coverslips, and all growth factors were diluted first into a 20X concentrated solution of 1mM HCl before the final dilution (resulting in a final concentration of 20 uM HCl in the medium). All factors were added once, at the time of plating. The concentration of insulin in the medium was decreased from 5 ug/ml to 2.5 ug/ml.

5 Cultures were plated in triplicate. Either GDNFR $\alpha$  or NTN $\alpha$  at 1 ug/ml was added to the cultures 2 hours after cell plating. At the same time, parallel cultures received either GDNFR $\alpha$  or NTN $\alpha$  plus 50 ng/ml of either GDNF or NTN, or received 50ng/ml of GDNF or NTN without exogenously added receptor. After 4 days in culture, the cells were fixed and stained for tyrosine hydroxylase (TH), a marker for dopaminergic neurons, and the number of TH+ cells in each condition was counted and compared to control cultures

10 (parallel cultures grown in the presence of no added factors). Both the GDNFR $\alpha$  and the NTN $\alpha$  extracellular domains contained a histidine tag (6 histidine residues) at the C-terminus, which provided a convenient handle for purification of the extracellular domains. The His-tagged-GDNFR $\alpha$  ECD C-terminal is DGLAGASSH HHHHH, where one of the His residues normally present in the GDNFR sequence was used to provide part

15 96 hr after transfection), purified over a Ni-NTA column using standard IMAC purification procedure. The isolated ECDs were dialyzed into PBS and subsequently stored at 4°C.

In three separate experiments GDNFR $\alpha$  and NTN $\alpha$  extracellular domains, in both the absence and presence of exogenously added ligand, promoted survival of dopaminergic neurons that was significantly greater than control cultures (Figures 8A to 8C). In each case, the amount of survival was equal or slightly

20 better than the amount of survival with ligand (GDNF or NTN) alone. Survival was further increased when both ligand and its particular receptor extracellular domain (e.g. NTN + NTN $\alpha$ , GDNF + GDNFR $\alpha$ ) were added together. Anti-NTN monoclonal antibody added to a control culture (with no added factors) did not result in a decrease in the observed background level of cell growth, indicating that background growth during the test period was not due to endogenous NTN, if present. These results indicate that receptor extracellular

25 domain addition of this family of receptors, without ligand, promotes a significant neuron survival effect over control.

#### Example 10

##### NTN $\alpha$ and NTN Enhance Dopamine Utilization In Vivo

It was determined that soluble NTN $\alpha$  enhances the effects of NTN in the intact adult rat nigrostriatal

30 dopaminergic system. Administration of NTN $\alpha$  together with NTN increases the ratio of DOPAC (dihydroxyphenylacetic acid, the principal dopamine metabolite in rat) to dopamine, which is indicative of functional upregulation of dopaminergic neurons. Adult Sprague-Dawley rats (295-345 g, n=23) were administered a single 2  $\mu$ l injection of hNTN (0.1  $\mu$ g), soluble his-tagged hNTN $\alpha$  (0.6  $\mu$ g), both hNTN (0.1  $\mu$ g) and soluble his-tagged hNTN $\alpha$  (0.6  $\mu$ g), or vehicle (4% mannitol, 10 mM HEPES) in the right striatum

35 at stereotaxic coordinates 0.5 mm anterior, 3.0 mm lateral to bregma and 4.5 mm ventral to the dura using a 10  $\mu$ l Hamilton syringe with 26s gauge needle. Seven days after surgery, tissue from selected brain regions was harvested for analysis of dopamine and dopamine metabolite content. Following decapitation of rats, the brains were rapidly removed and immersed in ice cold phosphate-buffered saline for 30 seconds, 1 mm coronal sections were cut with the aid of a chilled metal brain matrix, and tissue punches of three regions of the

striatum (anterior, central and posterior), nucleus accumbens, and substantia nigra were collected using 11, 13, and 16 gauge needles, respectively. Tissue punches were homogenized in 200  $\mu$ l 0.1 M perchloric acid containing DHBA as an internal standard. Homogenates were centrifuged at 23,000-28,000 x g for 20 minutes and the supernatants analyzed for dopamine and DOPAC content using ion-pair reverse-phase HPLC with electrochemical detection. The DOPAC / dopamine ratio was calculated on the injected and uninjected sides, and expressed as the injected side as a percentage of the uninjected side. As shown in Figure 9, the combination of NTN and NTN $\alpha$  increased DOPAC / dopamine ratio in the striatum compared to vehicle, NTN $\alpha$  alone, and NTN alone. These results show that a combination of NTN and NTN $\alpha$  can potentially enhance dopamine utilization in the striatum, which is useful in the treatment of Parkinson's disease.

Presented herein is a novel sequence encoding a novel receptor for neurturin, a recently discovered member of the GDNF protein family. The results presented herein reveal the existence of a novel family of multi-component receptors for growth and differentiation factors. The receptor complex is composed of a shared signaling subunit--the transmembrane tyrosine kinase receptor Ret--and a GPI-linked, receptor-specific-ligand-binding subunit--NTN $\alpha$  in the case of NTN, and GDNF $\alpha$  in the case of GDNF. In view of these findings, further details on the mechanism of action of NTN and GDNF on the molecular level can be determined. In addition, it is now clear that the distinct biological activities of the GDNF/NTN protein family are determined by the distinct tissue distribution of their respective ligand-binding-receptor components rather than by the ability to activate different signaling systems. Finally, the data presented herein provides a contrasting and simple biological rationale for the involvement of what previously appeared to be a needlessly complex way to activate a tyrosine kinase receptor (reviewed in Lindsay and Yancopoulos, *Neuron* 17:571-574 (1996)). It is now apparent that changing the ligand-binding accessory molecule is a most economic way to recruit the same signalling system for usage by multiple growth factors, and that GPI-linked proteins are economic ligand-binding accessory molecules. Use of a single transmembrane signalling system by multiple growth factors appears to be employed by cytokines (Stahl and Yancopoulos, *Ann. Rev. Biophys. Biomol. Struct.* 24:269-291 (1993)) as well as by members of the transforming growth factor protein family (Wrana *et al.*, *Nature* 370:341-347 (1994)). Likewise, GPI-linked proteins are used as ligand-binding accessory molecules in several multi-component receptors, such as the bacterial endotoxin receptor (Lee *et al.*, *Proc. Natl. Acad. Sci. USA* 90:9930-9934 (1993); Pugin *et al.*, *Proc. Natl. Acad. Sci. USA* 90:2744-2748 (1993)) and the receptor for ciliary neurotrophic factor (Davies *et al.*, *Science* 259:1736-1739 (1993)). The discovery of the receptor and associated receptor system presented herein defines a novel paradigm in signal transduction, highlights the diverse strategies which are used to transmit extracellular signals in the vertebrate nervous system, and provides means for modulating and controlling cell activity and survival that expand the treatment modalities available to the clinician.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Genentech, Inc.
- (ii) TITLE OF INVENTION: Neurturin Receptor
- 5 (iii) NUMBER OF SEQUENCES: 19
- (iv) CORRESPONDENCE ADDRESS:
- 10 (A) ADDRESSEE: Genentech, Inc.  
(B) STREET: 1 DNA Way  
(C) CITY: South San Francisco  
(D) STATE: California  
(E) COUNTRY: USA  
(F) ZIP: 94080
- (v) COMPUTER READABLE FORM:
- 15 (A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: WinPatin (Genentech)
- (vi) CURRENT APPLICATION DATA:
- 20 (A) APPLICATION NUMBER:  
(B) FILING DATE:  
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- 25 (A) APPLICATION NUMBER: 08/871913  
(B) FILING DATE: 9-Jun-1997
- (vii) PRIOR APPLICATION DATA:
- 30 (A) APPLICATION NUMBER: 08/802805  
(B) FILING DATE: 18-Feb-1997
- (vii) PRIOR APPLICATION DATA:
- 30 (A) APPLICATION NUMBER: 08/957063  
(B) FILING DATE: 24-Oct-1997
- (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Torchia, PhD., Timothy E.  
(B) REGISTRATION NUMBER: 36,700  
(C) REFERENCE/DOCKET NUMBER: P1086R3PCT
- 35 (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: 650/225-8674  
(B) TELEFAX: 650/952-9881
- (2) INFORMATION FOR SEQ ID NO:1:
- 40 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 2600 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
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## (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1392 base pairs  
(B) TYPE: Nucleic Acid  
20 (C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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 ATTGGGTTTG ACATGACACC TAACTATGTG GACTCCAGCC CCACTGGCAT 950  
 CGTGGTGTCC CCCTGGTGCA GCTGTCTGTG CAGCGGGAAC ATGGAGGAGG 1000  
 AGTGTGAGAA GTTCCTCAGG GACTTCACCG AGAACCCATG CCTCCGGAAC 1050  
 GCCATCCAGG CCTTTGGCAA CGGCACGGAC GTGAACGTGT CCCCCAAAGG 1100  
 10 CCCCTCGTTC CAGGCCACCC AGGCCCTCG GGTGGAGAAG ACGCCTTCTT 1150  
 TGCCAGATGA CCTCAGTGAC AGTACCAGCT TGGGGACCAG TGTCATCACC 1200  
 ACCTGCACGT CTGTCCAGGA GCAGGGGCTG AAGGCCAACA ACTCCAAAGA 1250  
 GTTAAGCATG TGCTTCACAG AGCTCACGAC AAATATCATC CCAGGGAGTA 1300  
 ACAAGGTGAT CAAACCTAAC TCAGGCCCCA GCAGAGCCAG ACCGTCGGCT 1350  
 15 GCCTTGACCG TGCTGTCTGT CCTGATGCTG AACTGGCCT TG 1392

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 464 amino acids  
 (B) TYPE: Amino Acid  
 20 (D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met	Ile	Leu	Ala	Asn	Val	Phe	Phe	Leu	Phe	Phe	Phe	Leu	Asp	Glu	1	5	10	15
Thr	Leu	Arg	Ser	Leu	Ala	Ser	Pro	Ser	Ser	Leu	Gln	Asp	Pro	Glu	20	25	30	
Leu	His	Gly	Trp	Arg	Pro	Pro	Val	Asp	Cys	Val	Arg	Ala	Asn	Glu	35	40	45	
Leu	Cys	Ala	Ala	Glu	Ser	Asn	Cys	Ser	Ser	Arg	Tyr	Arg	Thr	Leu	50	55	60	
Arg	Gln	Cys	Leu	Ala	Gly	Arg	Asp	Arg	Asn	Thr	Met	Leu	Ala	Asn	65	70	75	
Lys	Glu	Cys	Gln	Ala	Ala	Leu	Glu	Val	Leu	Gln	Glu	Ser	Pro	Leu	80	85	90	
Tyr	Asp	Cys	Arg	Cys	Lys	Arg	Gly	Met	Lys	Lys	Glu	Leu	Gln	Cys	95	100	105	
Leu	Gln	Ile	Tyr	Trp	Ser	Ile	His	Leu	Gly	Leu	Thr	Glu	Gly	Glu	110	115	120	
Glu	Phe	Tyr	Glu	Ala	Ser	Pro	Tyr	Glu	Pro	Val	Thr	Ser	Arg	Leu	125	130	135	



	Ser Asp Ile Phe Arg Leu Ala Ser Ile Phe Ser Gly Thr Gly Ala	140	145	150
	Asp Pro Val Val Ser Ala Lys Ser Asn His Cys Leu Asp Ala Ala	155	160	165
5	Lys Ala Cys Asn Leu Asn Asp Asn Cys Lys Lys Leu Arg Ser Ser	170	175	180
	Tyr Ile Ser Ile Cys Asn Arg Glu Ile Ser Pro Thr Glu Arg Cys	185	190	195
10	Asn Arg Arg Lys Cys His Lys Ala Leu Arg Gln Phe Phe Asp Arg	200	205	210
	Val Pro Ser Glu Tyr Thr Tyr Arg Met Leu Phe Cys Ser Cys Gln	215	220	225
	Asp Gln Ala Cys Ala Glu Arg Arg Arg Gln Thr Ile Leu Pro Ser	230	235	240
15	Cys Ser Tyr Glu Asp Lys Glu Lys Pro Asn Cys Leu Asp Leu Arg	245	250	255
	Gly Val Cys Arg Thr Asp His Leu Cys Arg Ser Arg Leu Ala Asp	260	265	270
20	Phe His Ala Asn Cys Arg Ala Ser Tyr Gln Thr Val Thr Ser Cys	275	280	285
	Pro Ala Asp Asn Tyr Gln Ala Cys Leu Gly Ser Tyr Ala Gly Met	290	295	300
	Ile Gly Phe Asp Met Thr Pro Asn Tyr Val Asp Ser Ser Pro Thr	305	310	315
25	Gly Ile Val Val Ser Pro Trp Cys Ser Cys Arg Gly Ser Gly Asn	320	325	330
	Met Glu Glu Glu Cys Glu Lys Phe Leu Arg Asp Phe Thr Glu Asn	335	340	345
30	Pro Cys Leu Arg Asn Ala Ile Gln Ala Phe Gly Asn Gly Thr Asp	350	355	360
	Val Asn Val Ser Pro Lys Gly Pro Ser Phe Gln Ala Thr Gln Ala	365	370	375
	Pro Arg Val Glu Lys Thr Pro Ser Leu Pro Asp Asp Leu Ser Asp	380	385	390
35	Ser Thr Ser Leu Gly Thr Ser Val Ile Thr Thr Cys Thr Ser Val	395	400	405
	Gln Glu Gln Gly Leu Lys Ala Asn Asn Ser Lys Glu Leu Ser Met	410	415	420
40	Cys Phe Thr Glu Leu Thr Thr Asn Ile Ile Pro Gly Ser Asn Lys	425	430	435
	Val Ile Lys Pro Asn Ser Gly Pro Ser Arg Ala Arg Pro Ser Ala	440	445	450
	Ala Leu Thr Val Leu Ser Val Leu Met Leu Lys Leu Ala Leu	455	460	464

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 3358 base pairs  
(B) TYPE: Nucleic Acid  
5 (C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCGGTGGCGG CCGCTCTAGA ACTAGTGGAT CCCCgggCTG CAGGAATTCG 50  
GCACGAGAGT GAGCCGAGCA AGGGTTAGCG GGAGAAGATT TTTTTTTTTT 100  
10 TGAATCTTTT TCTTCGTCTT GGTGCCAAAG AAGCGACTCT GGTCTCCCGT 150  
CCTAGAAGCT CCTACTGGAT TGCTCCTATT CCGTCGGTGG ATTTCTTTCC 200  
TATTCGCATT TATTCTGACC CCCTCCCTCG CTGCTTCCTT CCAGCCCTTC 250  
ACTTTCAGAT CGCCTCGCCC CCACCTCTCC AGTCCCCTCC TGGGAAGTGC 300  
AGGGGAATTG GACCCACGGG GACTCACGCC TTCCCGGACG CGCGAGCAAA 350  
15 GGGCTGGGCT GACCTCAGGA CCAGGCTGTT GGCTTAGAAG GCAGCCAGAC 400  
ACATAGCTAC GTGTGTTTGA TTTCAGTGGC AAGGGGGGAC GTCGAGAGGC 450  
AGCCCACCGC CCGCCTCCTA CCCCTCCCCC TCCAACCAGC AGTGAGAATC 500  
CCAGGACTCG GGATCTTCAA CCGGCGGCCG CCCGGCGGGA TCTCCGCATT 550  
GGATTTGGGG GTCGTTATTG CTCGGCTGTT ATTATTATCG TTATTTTATT 600  
20 TTTATTTTTT AAACCTAAGG GAGAAAGACA CATAACACA AACTGTGGG 650  
ATTTATTTAA CATGATCTTG GCAAACGCCT TCTGCCTCTT CTTCTTTTAA 700  
GACGAAACCC TCCGCTCTTT GGCCAGCCCT TCCTCCCTGC AGGGCTCTGA 750  
GCTCCACGGC TGGCGCCCCC AAGTGGACTG TGTCCGGGCC AATGAGCTGT 800  
GTGCGGCTGA ATCCAAGTGC AGCTCCAGGT ACCGCACCTT TCGGCAGTGC 850  
25 CTGGCAGGCC GGGATCGCAA TACCATGCTG GCCAATAAGG AGTGCCAGGC 900  
AGCCCTGGAG GTCTTGAGG AAAGCCCACT GTATGACTGC CGCTGCAAGC 950  
GGGGCATGAA GAAGGAGCTG CAGTGTCTGC AGATCTACTG GAGCATCCAT 1000  
CTGGGGCTGA CAGAGGGTGA GGAGTTCTAT GAAGCTTCCC CCTATGAGCC 1050  
TGTGACCTCG CGCCTCTCGG ACATCTTCAG GCTCGCTTCA ATCTTCTCAG 1100  
30 GGACAGGGAC AGACCCGGCG GTCAGTACCA AAAGCAACCA CTGCCTGGAT 1150  
GCCGCCAAGG CCTGCAACCT GAATGACAAC TGCAAGAAGC TTCGCTCCTC 1200  
TTATATCTCC ATCTGCAACC GTGAGATCTC TCCCACCGAA CGCTGCAACC 1250  
GCCGCAAGTG CCACAAGGCT CTGCGCCAGT TCTTTGACCG TGTGCCCAGC 1300  
GAGTATACCT ACCGCATGCT CTTCTGCTCC TGTCAGGACC AGGCATGTGC 1350  
35 TGAGCGTCGC CGGCAAACCA TCCTGCCCAG TTGCTCCTAT GAGGACAAGG 1400

AGAAGCCCAA CTGCCTGGAC CTGCGCAGCC TGTGTCGTAC AGACCACCTG 1450  
TGCCGGTCCC GACTGGCAGA TTTCCACGCC AACTGTGAG CCTCCTACCG 1500  
GACAATCACC AGCTGTCCTG CGGACAACTA CCAGGCATGT CTGGGCTCCT 1550  
ATGCTGGCAT GATTGGGTTT GATATGACAC CCAACTATGT GGACTCCAAC 1600  
5 CCCACGGGCA TCGTGGTGTG TCCCTGGTGC AATTGTCTGT GCAGTGGGAA 1650  
CATGGAAGAA GAGTGTGAGA AGTTCCTCAG GGACTTCACG GAAAACCCAT 1700  
GCCTCCGGAA TGCCATTCAG GCCTTTGGTA ATGGCACAGA TGTGAACATG 1750  
TCTCCCAAAG GCCCCTCACT CCCAGCTACC CAGGCCCTC GGGTGGAGAA 1800  
GACTCCTTCA CTGCCAGATG ACCTCAGTGA CAGCACCAGC CTGGGGACCA 1850  
10 GTGTCATCAC CACCTGCACA TCTATCCAGG AGCAAGGGCT GAAGGCCAAC 1900  
AACTCCAAAG AGTTAAGCAT GTGCTTCACA GAGCTCACGA CAAACATCAG 1950  
TCCAGGGAGT AAAAAGGTGA TCAAACCTAA CTCAGGCTCC AGCAGAGCCA 2000  
GACTGTGCGC TGCCTTGACT GCCCTCCAC TCCTGATGCT GACCTTGCC 2050  
TTGTAGGCCT TTGGAACCA GCACAAAAGT TCTTCAAGCA ACCCAGATAT 2100  
15 GAACTCCCGC CTGACAAAAT GGAAACACAC GCATACACAC ATGCACACAC 2150  
ACACAAACAC ACACACACAC ACACACACAC ACACACACAC ACACACACAC 2200  
ACACCCCTTG CAAAAACACT TTTTTCCTA CATTGTCTCT GAACCTTCT 2250  
CCTCCCAAGT TTCTTCTCTG GAGAAGTTTT TCTAAACCA ACAGACAAGC 2300  
AGGCGGGCAG TCAGAAGCCT GCCCAGAGGT CCCCTGCAAG GGACACCCAG 2350  
20 CACCAACGAG GGCTCAAGGC TCTTGAGAGA CTCTTTTCTC TTCACTGGTG 2400  
TTTTCTCTCT GGACAAGATG AGACCCTGAT GTGGAAGGTA CTTTGCTGTG 2450  
CCTGGTGTGG ACTGGGGAAA GGACAGTTGC AGCTGCCTAC TCTGGGGACC 2500  
TGCCCAAGGG TTCACAGAGA GTCTCAGTCA GCAAGGAAGC AGGGCTGGCC 2550  
ACAAGGACTT TGTCACCTCT TCCTCTTGGC TTCAGAGATG GAAATGGTTT 2600  
25 GCTGCCATCC CCAGCCATTA TGTGGCCTAG TGGGTTTAAG TCTGGAGTAG 2650  
GAAGCCTCAT GGCAGCTTCA GGCCATGGTG CCTGTAGTAT AGCTGGGGTT 2700  
GGGAGCTGTT ACAGGAGGAA GCTTCTTTGG GGCATGAGCA AGCCTTGGTT 2750  
GGGCACCAGC TCCAAGATGT ACCTTCCTCC TTTATGCCAG GAATCTTGAA 2800  
GTCAAAGAGA AATGATCCTC TGTTGGCTCT TTTTGTGTTG TTTTGAATT 2850  
30 TTTTGTGGG TCCATTGGC AGGTCTCTCT TGGGGAGAAG GGCTGTTGAG 2900  
TGGGGCTGGG GAGACCCTAG CTGGGCGTGT GTATGGAGCA CTCTGGTGGG 2950  
TTCCCAAGCT TGCCCTTCT CTCTCTTGT TTCTGCTTC TCTCTCATTT 3000  
CTGAGACATT CATGCACTGT CTGTACATAC TGGGTCTCCT TTCTCAACAT 3050

ATGTGTATAT CCATATCCAT ATATCCTATG ATTTTACTCT TTCTTTCATT 3100  
TTTTTTAAAG AAACAAACT ATGGAAATAA TACCCTACAG ATGAGCGAAA 3150  
ATGTATTATT GTAAAGTTTA TTTTTTTTAA TAATGTTGTC TATGATGGGA 3200  
AGAAAGGTAC CAGGACCCCT GAGCCCTGGT CCAGTTGGGC TGGTGGGGCT 3250  
5 GTGGCCGGGG ACTCCCGATT GCATTCACTC TTAACCAAGC TCCAATAAAC 3300  
GTACTAGGAA GCGAAAAAAA AAAAAAAAAA ACTCGAGGGG GGGCCCGGTA 3350  
CCCAATTC 3358

(2) INFORMATION FOR SEQ ID NO:5:

- 10 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1392 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

15 ATGATCTTGG CAAACGCCTT CTGCCTCTTC TTCTTTTATAG ACGAAACCCT 50  
CCGCTCTTTG GCCAGCCCTT CCTCCCTGCA GGGCTCTGAG CTCCACGGCT 100  
GGCGCCCCCA AGTGGACTGT GTCCGGGCCA ATGAGCTGTG TCGGGCTGAA 150  
TCCAAGTGCA GCTCCAGGTA CCGCACCTT CCGCAGTGCC TGGCAGGCCG 200  
GGATCGCAAT ACCATGCTGG CCAATAAGGA GTGCCAGGCA GCCCTGGAGG 250  
20 TCTTGCAAGGA AAGCCCACTG TATGACTGCC GCTGCAAGCG GGGCATGAAG 300  
AAGGAGCTGC AGTGTCTGCA GATCTACTGG AGCATCCATC TGGGGCTGAC 350  
AGAGGGTGAG GAGTTCTATG AAGCTTCCCC CTATGAGCCT GTGACCTCGC 400  
GCCTCTCGGA CATCTTCAGG CTCGCTTCAA TCTTCTCAGG GACAGGGACA 450  
GACCCGGCGG TCAGTACCAA AAGCAACCAC TGCCTGGATG CCGCCAAGGC 500  
25 CTGCAACCTG AATGACAACT GCAAGAAGCT TCGCTCCTCT TATATCTCCA 550  
TCTGCAACCG TGAGATCTCT CCCACCGAAC GCTGCAACCG CCGCAAGTGC 600  
CACAAGGCTC TGCGCCAGTT CTTTGACCGT GTGCCCAGCG AGTATACCTA 650  
CCGCATGCTC TTCTGCTCCT GTCAGGACCA GGCATGTGCT GAGCGTCGCC 700  
GGCAAACCAT CCTGCCCAGT TGCTCCTATG AGGACAAGGA GAAGCCCAAC 750  
30 TGCCTGGACC TGCGCAGCCT GTGTGCTACA GACCACCTGT GCCGGTCCCG 800  
ACTGGCAGAT TTCCACGCCA ACTGTGAGC CTCCTACCGG ACAATCACCA 850  
GCTGTCCTGC GGACAACTAC CAGGCATGTC TGGGCTCCTA TGCTGGCATG 900  
ATTGGGTTTG ATATGACACC CAACTATGTG GACTCCAACC CCACGGGCAT 950  
CGTGGTGTCT CCCTGGTGCA ATTGTCGTGG CAGTGGGAAC ATGGAAGAAG 1000  
35 AGTGTGAGAA GTTCTCAGG GACTTCACGG AAAACCCATG CCTCCGGAAT 1050

GCCATTCAGG CCTTTGGTAA TGGCACAGAT GTGAACATGT CTCCCAAAGG 1100  
 CCCCTCACTC CCAGCTACCC AGGCCCTCG GGTGGAGAAG ACTCCTTCAC 1150  
 TGCCAGATGA CCTCAGTGAC AGCACCAGCC TGGGGACCAG TGTCATCACC 1200  
 ACCTGCACAT CTATCCAGGA GCAAGGGCTG AAGGCCAACA ACTCCAAAGA 1250  
 5 GTTAAGCATG TGCTTCACAG AGCTCACGAC AAACATCAGT CCAGGGAGTA 1300  
 AAAAGGTGAT CAAACTTAAC TCAGGCTCCA GCAGAGCCAG ACTGTCGGCT 1350  
 GCCTTGACTG CCCTCCCACT CCTGATGCTG ACCTTGGCCT TG 1392

## (2) INFORMATION FOR SEQ ID NO:6:

- 10 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 464 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

15	Met	Ile	Leu	Ala	Asn	Ala	Phe	Cys	Leu	Phe	Phe	Phe	Leu	Asp	Glu	1	5	10	15
	Thr	Leu	Arg	Ser	Leu	Ala	Ser	Pro	Ser	Ser	Leu	Gln	Gly	Ser	Glu	20	25	30	
	Leu	His	Gly	Trp	Arg	Pro	Gln	Val	Asp	Cys	Val	Arg	Ala	Asn	Glu	35	40	45	
20	Leu	Cys	Ala	Ala	Glu	Ser	Asn	Cys	Ser	Ser	Arg	Tyr	Arg	Thr	Leu	50	55	60	
	Arg	Gln	Cys	Leu	Ala	Gly	Arg	Asp	Arg	Asn	Thr	Met	Leu	Ala	Asn	65	70	75	
25	Lys	Glu	Cys	Gln	Ala	Ala	Leu	Glu	Val	Leu	Gln	Glu	Ser	Pro	Leu	80	85	90	
	Tyr	Asp	Cys	Arg	Cys	Lys	Arg	Gly	Met	Lys	Lys	Glu	Leu	Gln	Cys	95	100	105	
	Leu	Gln	Ile	Tyr	Trp	Ser	Ile	His	Leu	Gly	Leu	Thr	Glu	Gly	Glu	110	115	120	
30	Glu	Phe	Tyr	Glu	Ala	Ser	Pro	Tyr	Glu	Pro	Val	Thr	Ser	Arg	Leu	125	130	135	
	Ser	Asp	Ile	Phe	Arg	Leu	Ala	Ser	Ile	Phe	Ser	Gly	Thr	Gly	Thr	140	145	150	
35	Asp	Pro	Ala	Val	Ser	Thr	Lys	Ser	Asn	His	Cys	Leu	Asp	Ala	Ala	155	160	165	
	Lys	Ala	Cys	Asn	Leu	Asn	Asp	Asn	Cys	Lys	Lys	Leu	Arg	Ser	Ser	170	175	180	
	Tyr	Ile	Ser	Ile	Cys	Asn	Arg	Glu	Ile	Ser	Pro	Thr	Glu	Arg	Cys	185	190	195	
40	Asn	Arg	Arg	Lys	Cys	His	Lys	Ala	Leu	Arg	Gln	Phe	Phe	Asp	Arg	200	205	210	
	Val	Pro	Ser	Glu	Tyr	Thr	Tyr	Arg	Met	Leu	Phe	Cys	Ser	Cys	Gln				

	215	220	225
	Asp Gln Ala Cys Ala Glu Arg Arg Arg Gln Thr Ile Leu Pro Ser		
	230	235	240
5	Cys Ser Tyr Glu Asp Lys Glu Lys Pro Asn Cys Leu Asp Leu Arg		
	245	250	255
	Ser Leu Cys Arg Thr Asp His Leu Cys Arg Ser Arg Leu Ala Asp		
	260	265	270
	Phe His Ala Asn Cys Arg Ala Ser Tyr Arg Thr Ile Thr Ser Cys		
	275	280	285
10	Pro Ala Asp Asn Tyr Gln Ala Cys Leu Gly Ser Tyr Ala Gly Met		
	290	295	300
	Ile Gly Phe Asp Met Thr Pro Asn Tyr Val Asp Ser Asn Pro Thr		
	305	310	315
15	Gly Ile Val Val Ser Pro Trp Cys Asn Cys Arg Gly Ser Gly Asn		
	320	325	330
	Met Glu Glu Glu Cys Glu Lys Phe Leu Arg Asp Phe Thr Glu Asn		
	335	340	345
	Pro Cys Leu Arg Asn Ala Ile Gln Ala Phe Gly Asn Gly Thr Asp		
	350	355	360
20	Val Asn Met Ser Pro Lys Gly Pro Ser Leu Pro Ala Thr Gln Ala		
	365	370	375
	Pro Arg Val Glu Lys Thr Pro Ser Leu Pro Asp Asp Leu Ser Asp		
	380	385	390
25	Ser Thr Ser Leu Gly Thr Ser Val Ile Thr Thr Cys Thr Ser Ile		
	395	400	405
	Gln Glu Gln Gly Leu Lys Ala Asn Asn Ser Lys Glu Leu Ser Met		
	410	415	420
	Cys Phe Thr Glu Leu Thr Thr Asn Ile Ser Pro Gly Ser Lys Lys		
	425	430	435
30	Val Ile Lys Leu Asn Ser Gly Ser Ser Arg Ala Arg Leu Ser Ala		
	440	445	450
	Ala Leu Thr Ala Leu Pro Leu Leu Met Leu Thr Leu Ala Leu		
	455	460	464

## (2) INFORMATION FOR SEQ ID NO:7:

- 35 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 229 base pairs
  - (B) TYPE: Nucleic Acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Linear

- 40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCAAGAGCAA CCATTGCCTG GATGCTGCCA AGGCCTGCAA CCTGAATGAC 50  
 AACTGCAAGA AGCTGCGCTC CTCCTACATC TCCATCTGCA ACCGCGAGAT 100  
 CTCGCCCACC GAGCGCTGCA ACCGCCGCAA GTGCCACAAG GCCCTGCGCC 150

AGTTCTTCGA CCGGGTGCCC AGCGAGTACA CCTACCGCAT GCTCTTCTGC 200  
TCCTGCCAAG ATCAGGCGTG CGCTGAGNC 229

(2) INFORMATION FOR SEQ ID NO:8:

- 5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 521 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

10 CAACCATTGC CTGGGATGCT GCCAAGGCCT GCAACCTGAA TGACAACTGC 50  
AAGAAGCTGC GTCCTCCTA CATCTCCATC TGCAACCGCG AGATCTCGCC 100  
CACCGAGCGC TGCAACCGCC GCAAGTGCCA CAAGGCCCTG CGCCAGTTCT 150  
TCGACCGGGT GCCCAGCGAG TACACCTACC GCATGCTCTT CTGCTCCTGC 200  
CAAGACCAGG CGTGCGCTGA GCCGCGGNCA AAACCATCCT GCCCAGCTGC 250  
15 TCCTATGAGG ACAAGGAGAA GCCCAACTGC CTGGGACCTG CGTGGCGTGT 300  
GCCGGGACTG ACCACCTGTG TCGGTCCCGG CTNGGCCGAC TTTCCATGGC 350  
CAATTTGTTG GAGCCTTCCT ACCAGACGGG TCANCAGGTT GCCTTGCGGA 400  
CAATTTACCA GGGGTNTTTT GGGGTTTTTA TTGTTGGGCA TGGATTGGGG 450  
TTTTGAAATT GANAATTAAT TTTGTTGGGA TTTNCAGGCC CCATTGGGCN 500  
20 TTGTNGGTGN TTCCCCTGGG G 521

(2) INFORMATION FOR SEQ ID NO:9:

- 25 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 478 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TGACACCTAA CTATGTGGAC TCCAGCCCCA CTGGCATCGT GGTGTCCCCC 50  
TGGTGACGCT GTCGTGGCAG CGGGAACATG GAGGAGGAGT GTGAGAAGTT 100  
30 CCTCAGGGAC TTCACCGAGA ACCCATGCCT CCGGAACGCC ATCCAGGCCT 150  
TTGNAACGGC ACGGACGTGA ACGTGTCCCC AAAAGGCCCC TCGTTCCAGG 200  
CCACCCAGGC CCTCGGGTGG AGAAGACGCC TTCTTTGCCA GATGACCTCA 250  
GTGACAGTAC CAGCTTGGGG ACCAGTGTCA TCACCACCTG CACGTCTGTC 300  
CAGGAGCAGG GGCTGAAGGC CAACAACTCC AAAGAGTTAA GCATGTGCTT 350  
35 CACAGAGCTC ACCGACAAAT ATCATCCCAG GGAGTAACAA GGTGATTCAA 400  
ACCTAACTCA GGCCCCAGCA GAGCAAGACC GTCGGCTTGC CTTTGACCGT 450  
GCTGTCTGTC CTGATGCTGA ACAGGCTT 478

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 433 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCAAGGTGTG TGTGTGTCTG TGTGTGTTTC CATTTCGTCA GGCGGCTGTT 50  
CTTGTCTGCG TACTTTCAAA AATCTTCTGA CTCGGTTCCC ACAGCCTACA 100  
10 AGGCCTGTTT CAGCATCAGG ACAGACAGCA CGGTCAAGGC AGCCGACGGT 150  
CTGGCTCTGC TGGGGCCTGA GTTAGGTTTG ATCACCTTGT TACTCCCTGG 200  
GATGATATTT GTCGTGAGCT CTGTGAAGCA CATGCTTAAC TCTTTGGAGT 250  
TGTTGGCCTT CAGCCCCTGC TCCTGGACAG ACGTGCAGGT GGTGATGACA 300  
CTGGGTCCCC AAGCTGGTAC TGTCCTGAG GTCATCTGGC AAAGAAGGCG 350  
15 TCTTCTCCAC CCGAGGGGCC TGGGGTGGCT GGGAACGAGG GGGCCTTTTT 400  
GGGGGACACG TTCACGTTCC GTTGCCGTG CCA 433

## (2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 418 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TTTTTTTTTT TGGGAAAAC AATTTTTTTT TTGCAAGGTG TGTGTGTGTC 50  
25 TGTGTGTGTT TCCATTCGT CAGGCGGCTG TTCTTGTCTG CGTANTTTTC 100  
AAAAATCTTC TGA CTGGTT CCCACAGCCT ACAAGGCCTG TTTCAGCATC 150  
AGGACAGACA GCACGGTCAA GGCAGCCGAC GGTCTGGCTC TGCTGGGGCC 200  
TGAGTTAGGT TTGATCACCT TGTTACTCCC TGGGATGATA TTTNTCGTGA 250  
GCTCTGTGAA GCACATGCTT AACTCTTTGG AGTTNTTGGC CTTTCAGCCCC 300  
30 TGCTCCTGGG ACAGAACGTG CAGGNTGGGT GATGACACTG GGNCCCCAAG 350  
GCTGGGTACT GTCCTGAGG GTCATCTGGN CAAAGNAAGG NCGTTTTTCT 400  
CCACCCGAGG GGCCGGGG 418

## (2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 364 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:



TGTGTGTGTC TGTGTGTGTT TCCATTTCTG CAGGCGGCTG TTCTTGCTG 50  
 CGTAGTTTCA AAAATCTTCT GACTCGGTTC CCACAGCCTA CAAGGNCTGT 100  
 TTCAGCATCA GGACAGACAG CACGGTCAAG GCAGCCGACG GTCTGGCTCT 150  
 GCTGGGGCCT GAGTTAGGTT TGATCACCTT GTTACTCCCT GGGATGATAT 200  
 5 TTGTCGTGAG CTCTGTGAAG CACATGCTTA ACTCTTTGGA GTTGTGGCC 250  
 TTCAGCCCCT GCTCCTGGAC AGACGTGCAG GTGGTNATGA CACTGGTCCC 300  
 CAAGCTGGTA CTNTCACTGA GGTCATCTGG CAAAGAAGGC GTCTTCTCCA 350  
 CCCNAGGGGC CTGG 364

## (2) INFORMATION FOR SEQ ID NO:13:

- 10 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 319 base pairs
  - (B) TYPE: Nucleic Acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Linear

- 15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGGAAAAACA ATTTTATTTT TGCAAGGTGT GTGTGTGTCT GTGTGTGTTT 50  
 CCATTTCTGTC AGGCGGCTGT CCTTGTCTGC GTAGTTTCAA AAATCTTCTG 100  
 ACTCGGTTCC CACAGCCTAC AAGGCCTGTA TAAGCATCAG GACAGACAGC 150  
 ACGGTCAAGG CAGCCGACGG TCTGGCTCTG CTGGGGCCTG AGTAAGGTTT 200  
 20 GNCCACCTTG TAACTCCCTG GGATGATATT TGTCGTGAGC NCTGTNANGC 250  
 ACATGNTTAA CTCTTTGGAG TTNTTGGCCT TCAGCCCCTG CCCCTGGNCA 300  
 GACGTGCAGG TGGTGATGA 319

## (2) INFORMATION FOR SEQ ID NO:14:

- 25 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 309 base pairs
  - (B) TYPE: Nucleic Acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Linear

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

30 GCTGAAACTG GCCTTGTAGG CTGTGGGAAC CGAGTCAGAA TATTTTGTAA 50  
 AGCTACGCAG ACAAGAACNG CGGCCTGACG AAATGGAAAC ACACACAGAC 100  
 ACACACACNC CTTGCATAAA AAAAATTGTT TTTCCACCT TGTCGCTGAA 150  
 CCTGTCTCCT CCCAGGTTTC TTCTCTGGAG AAGTTTTTGT AAACCAAACA 200  
 GACAAGCAGG CAGGCAGCCT GAGAGCTGGC CCAGGGGTCC CCTGGTCAGG 250  
 35 GGAAACTCTG GTGCCGGGGA GGGCACGTGG CTCTAGAAAT GCCCTTCACT 300  
 TTCTCCTGG 309

## (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1995 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATGATCTTGG CAAACGTCTT CTTCTCTTC TTCTTTCTAG ACGAGACCCT 50  
CCGCTCTTTG GCCAGCCCTT CCTCCCTGCA GGACCCCGAG CTCCACGGCT 100  
GGCGCCCCC AGTGGACTGT GTCCGGGCCA ATGAGCTGTG TGCCGCCGAA 150  
10 TCCAAC TGCA GCTCTCGCTA CCGCACTCTG CGGCAGTGCC TGGCAGGCCG 200  
CGACCGCAAC ACCATGCTGG CCAACAAGGA GTGCCAGGCG GCCTTGGAGG 250  
TCTTGCAAGG GAGCCCGCTG TACGACTGCC GCTGCAAGCG GGGCATGAAG 300  
AAGGAGCTGC AGTGTCTGCA GATCTACTGG AGCATCCACC TGGGGCTGAC 350  
CGAGGGTGAG GAGTTCTACG AAGCCTCCCC CTATGAGCCG GTGACCTCCC 400  
15 GCCTCTCGGA CATCTTCAGG CTTGCTTCAA TCTTCTCAGG GACAGGGGCA 450  
GACCCGGTGG TCAGCGCCAA GAGCAACCAT TGCCTGGATG CTGCCAAGGC 500  
CTGCAACCTG AATGACAAC T GCAAGAAGCT GCGCTCCTCC TACATCTCCA 550  
TCTGCAACCG CGAGATCTCG CCCACCGAGC GCTGCAACCG CCGCAAGTGC 600  
CACAAGGCCC TGCGCCAGTT CTTGACCGG GTGCCCAGCG AGTACACCTA 650  
20 CCGCATGCTC TTCTGCTCCT GCCAAGACCA GCGTGCGCT GAGCGCCGCC 700  
GGCAAACCAT CCTGCCCAGC TGCTCCTATG AGGACAAGGA GAAGCCCAAC 750  
TGCCTGGACC TGCCTGGCGT GTGCCGGACT GACCACCTGT GTCGGTCCCG 800  
GCTGGCCGAC TTCCATGCCA ATTGTGAGC CTCCTACCAG ACGGTCACCA 850  
GCTGCCCTGC GGACAATTAC CAGGCGTGTC TGGGCTCTTA TGCTGGCATG 900  
25 ATTGGGTTT ACATGACACC TAACTATGTG GACTCCAGCC CCACTGGCAT 950  
CGTGGTGTCC CCCTGGTGCA GCTGTCGTGG CAGCGGGAAC ATGGAGGAGG 1000  
AGTGTGAGAA GTTCCTCAGG GACTTCACCG AGAACCCATG CCTCCGGAAC 1050  
GCCATCCAGG CCTTTGGCAA CGGCACGGAC GTGAACGTGT CCCCAAAAGG 1100  
CCCCTCGTTC CAGGCCACCC AGGCCCTCG GGTGGAGAAG ACGCCTTCTT 1150  
30 TGCCAGATGA CCTCAGTGAC AGTACCAGCT TGGGGACCAG TGTCATCACC 1200  
ACCTGCACGT CTGTCCAGGA GCAGGGGCTG AAGGCCAACA ACTCCAAAGA 1250  
GTTAAGCATG TGCTTCACAG AGCTCACGAC AAATATCATC CCAGGGCCTA 1300  
GGGACCCGGT GGACAAAAC CACACATGCC CACCGTGCCC AGCACCTGAA 1350  
CTCCTGGGGG GACCGTCAGT CTTCTCTTC CCCCCAAAAC CCAAGGACAC 1400  
35 CCTCATGATC TCCCGGACCC CTGAGGTCAC ATGCGTGGTG GTGGACGTGA 1450

GCCACGAAGA CCCTGAGGTC AAGTTCAACT GGTACGTGGA CGGCGTGGAG 1500  
 GTGCATAATG CCAAGACAAA GCCGCGGGAG GAGCAGTACA ACAGCACGTA 1550  
 CCGTGTGGTC AGCGTCCTCA CCGTCCTGCA CCAGGACTGG CTGAATGGCA 1600  
 AGGAGTACAA GTGCAAGGTC TCCAACAAAG CCCTCCCAGC CCCCATCGAG 1650  
 5 AAAACCATCT CCAAAGCCAA AGGGCAGCCC CGAGAACCAC AGGTGTACAC 1700  
 CCTGCCCCCA TCCCGGGAAG AGATGACCAA GAACCAGGTC AGCCTGACCT 1750  
 GCCTGGTCAA AGGCTTCTAT CCCAGCGACA TCGCCGTGGA GTGGGAGAGC 1800  
 AATGGGCAGC CGGAGAACAA CTACAAGACC ACGCCTCCCG TGCTGGACTC 1850  
 CGACGGCTCC TTCTTCCTCT ACAGCAAGCT CACCGTGGAC AAGAGCAGGT 1900  
 10 GGCAGCAGGG GAACGTCTTC TCATGCTCCG TGATGCATGA GGCTCTGCAC 1950  
 AACCCTACA CGCAGAAGAG CCTCTCCCTG TCTCCGGGTA AATGA 1995

## (2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 664 amino acids  
 15 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

	Met	Ile	Leu	Ala	Asn	Val	Phe	Phe	Leu	Phe	Phe	Phe	Leu	Asp	Glu	
	1				5					10					15	
20	Thr	Leu	Arg	Ser	Leu	Ala	Ser	Pro	Ser	Ser	Leu	Gln	Asp	Pro	Glu	
					20					25					30	
	Leu	His	Gly	Trp	Arg	Pro	Pro	Val	Asp	Cys	Val	Arg	Ala	Asn	Glu	
					35					40					45	
25	Leu	Cys	Ala	Ala	Glu	Ser	Asn	Cys	Ser	Ser	Arg	Tyr	Arg	Thr	Leu	
					50					55					60	
	Arg	Gln	Cys	Leu	Ala	Gly	Arg	Asp	Arg	Asn	Thr	Met	Leu	Ala	Asn	
					65					70					75	
	Lys	Glu	Cys	Gln	Ala	Ala	Leu	Glu	Val	Leu	Gln	Glu	Ser	Pro	Leu	
					80					85					90	
30	Tyr	Asp	Cys	Arg	Cys	Lys	Arg	Gly	Met	Lys	Lys	Glu	Leu	Gln	Cys	
					95					100					105	
	Leu	Gln	Ile	Tyr	Trp	Ser	Ile	His	Leu	Gly	Leu	Thr	Glu	Gly	Glu	
					110					115					120	
35	Glu	Phe	Tyr	Glu	Ala	Ser	Pro	Tyr	Glu	Pro	Val	Thr	Ser	Arg	Leu	
					125					130					135	
	Ser	Asp	Ile	Phe	Arg	Leu	Ala	Ser	Ile	Phe	Ser	Gly	Thr	Gly	Ala	
					140					145					150	
	Asp	Pro	Val	Val	Ser	Ala	Lys	Ser	Asn	His	Cys	Leu	Asp	Ala	Ala	
					155					160					165	
40	Lys	Ala	Cys	Asn	Leu	Asn	Asp	Asn	Cys	Lys	Lys	Leu	Arg	Ser	Ser	
					170					175					180	

	Tyr	Ile	Ser	Ile	Cys	Asn	Arg	Glu	Ile	Ser	Pro	Thr	Glu	Arg	Cys	185	190	195
	Asn	Arg	Arg	Lys	Cys	His	Lys	Ala	Leu	Arg	Gln	Phe	Phe	Asp	Arg	200	205	210
5	Val	Pro	Ser	Glu	Tyr	Thr	Tyr	Arg	Met	Leu	Phe	Cys	Ser	Cys	Gln	215	220	225
	Asp	Gln	Ala	Cys	Ala	Glu	Arg	Arg	Arg	Gln	Thr	Ile	Leu	Pro	Ser	230	235	240
10	Cys	Ser	Tyr	Glu	Asp	Lys	Glu	Lys	Pro	Asn	Cys	Leu	Asp	Leu	Arg	245	250	255
	Gly	Val	Cys	Arg	Thr	Asp	His	Leu	Cys	Arg	Ser	Arg	Leu	Ala	Asp	260	265	270
	Phe	His	Ala	Asn	Cys	Arg	Ala	Ser	Tyr	Gln	Thr	Val	Thr	Ser	Cys	275	280	285
15	Pro	Ala	Asp	Asn	Tyr	Gln	Ala	Cys	Leu	Gly	Ser	Tyr	Ala	Gly	Met	290	295	300
	Ile	Gly	Phe	Asp	Met	Thr	Pro	Asn	Tyr	Val	Asp	Ser	Ser	Pro	Thr	305	310	315
20	Gly	Ile	Val	Val	Ser	Pro	Trp	Cys	Ser	Cys	Arg	Gly	Ser	Gly	Asn	320	325	330
	Met	Glu	Glu	Glu	Cys	Glu	Lys	Phe	Leu	Arg	Asp	Phe	Thr	Glu	Asn	335	340	345
	Pro	Cys	Leu	Arg	Asn	Ala	Ile	Gln	Ala	Phe	Gly	Asn	Gly	Thr	Asp	350	355	360
25	Val	Asn	Val	Ser	Pro	Lys	Gly	Pro	Ser	Phe	Gln	Ala	Thr	Gln	Ala	365	370	375
	Pro	Arg	Val	Glu	Lys	Thr	Pro	Ser	Leu	Pro	Asp	Asp	Leu	Ser	Asp	380	385	390
30	Ser	Thr	Ser	Leu	Gly	Thr	Ser	Val	Ile	Thr	Thr	Cys	Thr	Ser	Val	395	400	405
	Gln	Glu	Gln	Gly	Leu	Lys	Ala	Asn	Asn	Ser	Lys	Glu	Leu	Ser	Met	410	415	420
	Cys	Phe	Thr	Glu	Leu	Thr	Thr	Asn	Ile	Ile	Pro	Gly	Pro	Arg	Asp	425	430	435
35	Pro	Val	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	440	445	450
	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	455	460	465
40	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	470	475	480
	Val	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	485	490	495
	Val	Asp	Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	500	505	510

	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	
					515					520					525	
	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	
					530					535					540	
5	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	
					545					550					555	
	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	
					560					565					570	
10	Ser	Arg	Glu	Glu	Met	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr	Cys	Leu	
					575					580					585	
	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	Val	Glu	Trp	Glu	Ser	
					590					595					600	
	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	Pro	Pro	Val	Leu	
					605					610					615	
15	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	Leu	Thr	Val	Asp	
					620					625					630	
	Lys	Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	Cys	Ser	Val	Met	
					635					640					645	
20	His	Glu	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Leu	Ser	Leu	
					650					655					660	
	Ser	Pro	Gly	Lys												
					664											

## (2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
- 25 (A) LENGTH: 1995 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

30 ATGATCTTGG CAAACGCCTT CTGCCTCTTC TTCTTTT TAG ACGAAACCCT 50

CCGCTCTTTG GCCAGCCCTT CCTCCCTGCA GGGCTCTGAG CTCCACGGCT 100

GGCGCCCCCA AGTGGACTGT GTCCGGGCCA ATGAGCTGTG TGCGGCTGAA 150

TCCAACTGCA GCTCCAGGTA CCGCACCTT CGGCAGTGCC TGGCAGGCCG 200

GGATCGCAAT ACCATGCTGG CCAATAAGGA GTGCCAGGCA GCCCTGGAGG 250

35 TCTTGCAAGGA AAGCCCACTG TATGACTGCC GCTGCAAGCG GGGCATGAAG 300

AAGGAGCTGC AGTGTCTGCA GATCTACTGG AGCATCCATC TGGGGCTGAC 350

AGAGGGTGAG GAGTTCTATG AAGCTTCCCC CTATGAGCCT GTGACCTCGC 400

GCCTCTCGGA CATCTTCAGG CTCGCTTCAA TCTTCTCAGG GACAGGGACA 450

GACCCGGCGG TCAGTACCAA AAGCAACCAC TGCCTGGATG CCGCCAAGGC 500

40 CTGCAACCTG AATGACAACT GCAAGAAGCT TCGCTCCTCT TATATCTCCA 550

TCTGCAACCG TGAGATCTCT CCCACCGAAC GCTGCAACCG CCGCAAGTGC 600

CACAAGGCTC TSCGCCAGTT CTTTGACCGT GTGCCCAGCG AGTATACCTA 650  
 CCGCATGCTC TTCTGCTCCT GTCAGGACCA GGCATGTGCT GAGCGTCGCC 700  
 GGCAAACCAT CCTGCCCAGT TGCTCCTATG AGGACAAGGA GAAGCCCAAC 750  
 TGCCTGGACC TGCGCAGCCT GTGTCGTACA GACCACCTGT GCCGGTCCCC 800  
 5 ACTGGCAGAT TTCCACGCCA ACTGTCGAGC CTCCTACCGG ACAATCACCA 850  
 GCTGTCCTGC GGACAACTAC CAGGCATGTC TGGGCTCCTA TGCTGGCATG 900  
 ATTGGGTTTG ATATGACACC CAACTATGTG GACTCCAACC CCACGGGCAT 950  
 CGTGGTGTCT CCCTGGTGCA ATTGTCGTGG CAGTGGGAAC ATGGAAGAAG 1000  
 AGTGTGAGAA GTTCCTCAGG GACTTCACGG AAAACCCATG CCTCCGGAAT 1050  
 10 GCCATTCAAG CCTTTGGTAA TGGCACAGAT GTGAACATGT CTCCCAAAGG 1100  
 CCCCTCACTC CCAGCTACCC AGGCCCTCG GGTGGAGAAG ACTCCTTCAC 1150  
 TGCCAGATGA CCTCAGTGAC AGCACCAGCC TGGGGACCAG TGTCATCACC 1200  
 ACCTGCACAT CTATCCAGGA GCAAGGGCTG AAGGCCAACA ACTCCAAAGA 1250  
 GTTAAGCATG TGCTTCACAG AGCTCACGAC AAACATCAGT CCAGGGTCTA 1300  
 15 GAGACCCGGT GGACAAAAC CACACATGCC CACCGTGCCC AGCACCTGAA 1350  
 CTCCTGGGGG GACCGTCAGT CTTCTCTTC CCCCCAAAC CCAAGGACAC 1400  
 CCTCATGATC TCCCGGACCC CTGAGGTAC ATGCGTGGTG GTGGACGTGA 1450  
 GCCACGAAGA CCCTGAGGTC AAGTTCAACT GGTACGTGGA CGGCGTGGAG 1500  
 GTGCATAATG CCAAGACAAA GCCGCGGGAG GAGCAGTACA ACAGCACGTA 1550  
 20 CCGTGTGGTC AGCGTCCTCA CCGTCCTGCA CCAGGACTGG CTGAATGGCA 1600  
 AGGAGTACAA GTGCAAGGTC TCCAACAAAG CCCTCCCAGC CCCCATCGAG 1650  
 AAAACCATCT CCAAAGCCAA AGGGCAGCCC CGAGAACCAC AGGTGTACAC 1700  
 CCTGCCCCCA TCCCGGGAAG AGATGACCAA GAACCAGGTC AGCCTGACCT 1750  
 GCCTGGTCAA AGGCTTCTAT CCCAGCGACA TCGCCGTGGA GTGGGAGAGC 1800  
 25 AATGGGCAGC CGGAGAACAA CTACAAGACC ACGCCTCCCG TGCTGGACTC 1850  
 CGACGGCTCC TTCTTCCTCT ACAGCAAGCT CACCGTGGAC AAGAGCAGGT 1900  
 GGCAGCAGGG GAACGTCTTC TCATGCTCCG TGATGCATGA GGCTCTGCAC 1950  
 AACCCTACA CGCAGAAGAG CCTCTCCCTG TCTCCGGGTA AATGA 1995

(2) INFORMATION FOR SEQ ID NO:18:

- 30 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 664 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

35 Met Ile Leu Ala Asn Ala Phe Cys Leu Phe Phe Phe Leu Asp Glu

	1	5	10	15
	Thr	Leu Arg Ser	Leu Ala Ser Pro Ser	Ser Leu Gln Gly Ser Glu
		20	25	30
5	Leu His Gly Trp	Arg Pro Gln Val Asp	Cys Val Arg Ala Asn	Glu
		35	40	45
	Leu Cys Ala Ala	Glu Ser Asn Cys Ser	Ser Arg Tyr Arg Thr	Leu
		50	55	60
	Arg Gln Cys Leu	Ala Gly Arg Asp Arg	Asn Thr Met Leu Ala	Asn
		65	70	75
10	Lys Glu Cys Gln	Ala Ala Leu Glu Val	Leu Gln Glu Ser Pro	Leu
		80	85	90
	Tyr Asp Cys Arg	Cys Lys Arg Gly Met	Lys Lys Glu Leu Gln	Cys
		95	100	105
15	Leu Gln Ile Tyr	Trp Ser Ile His Leu	Gly Leu Thr Glu Gly	Glu
		110	115	120
	Glu Phe Tyr Glu	Ala Ser Pro Tyr Glu	Pro Val Thr Ser Arg	Leu
		125	130	135
	Ser Asp Ile Phe	Arg Leu Ala Ser Ile	Phe Ser Gly Thr Gly	Thr
		140	145	150
20	Asp Pro Ala Val	Ser Thr Lys Ser Asn	His Cys Leu Asp Ala	Ala
		155	160	165
	Lys Ala Cys Asn	Leu Asn Asp Asn Cys	Lys Lys Leu Arg Ser	Ser
		170	175	180
25	Tyr Ile Ser Ile	Cys Asn Arg Glu Ile	Ser Pro Thr Glu Arg	Cys
		185	190	195
	Asn Arg Arg Lys	Cys His Lys Ala Leu	Arg Gln Phe Phe Asp	Arg
		200	205	210
	Val Pro Ser Glu	Tyr Thr Tyr Arg Met	Leu Phe Cys Ser Cys	Gln
		215	220	225
30	Asp Gln Ala Cys	Ala Glu Arg Arg Arg	Gln Thr Ile Leu Pro	Ser
		230	235	240
	Cys Ser Tyr Glu	Asp Lys Glu Lys Pro	Asn Cys Leu Asp Leu	Arg
		245	250	255
35	Ser Leu Cys Arg	Thr Asp His Leu Cys	Arg Ser Arg Leu Ala	Asp
		260	265	270
	Phe His Ala Asn	Cys Arg Ala Ser Tyr	Arg Thr Ile Thr Ser	Cys
		275	280	285
	Pro Ala Asp Asn	Tyr Gln Ala Cys Leu	Gly Ser Tyr Ala Gly	Met
		290	295	300
40	Ile Gly Phe Asp	Met Thr Pro Asn Tyr	Val Asp Ser Asn Pro	Thr
		305	310	315
	Gly Ile Val Val	Ser Pro Trp Cys Asn	Cys Arg Gly Ser Gly	Asn
		320	325	330
	Met Glu Glu Glu	Cys Glu Lys Phe Leu	Arg Asp Phe Thr Glu	Asn

	335	340	345
	Pro Cys Leu Arg Asn Ala Ile Gln Ala	Phe Gly Asn Gly Thr Asp	
	350	355	360
5	Val Asn Met Ser Pro Lys Gly Pro Ser	Leu Pro Ala Thr Gln Ala	
	365	370	375
	Pro Arg Val Glu Lys Thr Pro Ser Leu	Pro Asp Asp Leu Ser Asp	
	380	385	390
	Ser Thr Ser Leu Gly Thr Ser Val Ile	Thr Thr Cys Thr Ser Ile	
	395	400	405
10	Gln Glu Gln Gly Leu Lys Ala Asn Asn	Ser Lys Glu Leu Ser Met	
	410	415	420
	Cys Phe Thr Glu Leu Thr Thr Asn Ile	Ser Pro Gly Ser Arg Asp	
	425	430	435
15	Pro Val Asp Lys Thr His Thr Cys Pro	Pro Cys Pro Ala Pro Glu	
	440	445	450
	Leu Leu Gly Gly Pro Ser Val Phe Leu	Phe Pro Pro Lys Pro Lys	
	455	460	465
	Asp Thr Leu Met Ile Ser Arg Thr Pro	Glu Val Thr Cys Val Val	
	470	475	480
20	Val Asp Val Ser His Glu Asp Pro Glu	Val Lys Phe Asn Trp Tyr	
	485	490	495
	Val Asp Gly Val Glu Val His Asn Ala	Lys Thr Lys Pro Arg Glu	
	500	505	510
25	Glu Gln Tyr Asn Ser Thr Tyr Arg Val	Val Ser Val Leu Thr Val	
	515	520	525
	Leu His Gln Asp Trp Leu Asn Gly Lys	Glu Tyr Lys Cys Lys Val	
	530	535	540
	Ser Asn Lys Ala Leu Pro Ala Pro Ile	Glu Lys Thr Ile Ser Lys	
	545	550	555
30	Ala Lys Gly Gln Pro Arg Glu Pro Gln	Val Tyr Thr Leu Pro Pro	
	560	565	570
	Ser Arg Glu Glu Met Thr Lys Asn Gln	Val Ser Leu Thr Cys Leu	
	575	580	585
35	Val Lys Gly Phe Tyr Pro Ser Asp Ile	Ala Val Glu Trp Glu Ser	
	590	595	600
	Asn Gly Gln Pro Glu Asn Asn Tyr Lys	Thr Thr Pro Pro Val Leu	
	605	610	615
	Asp Ser Asp Gly Ser Phe Phe Leu Tyr	Ser Lys Leu Thr Val Asp	
	620	625	630
40	Lys Ser Arg Trp Gln Gln Gly Asn Val	Phe Ser Cys Ser Val Met	
	635	640	645
	His Glu Ala Leu His Asn His Tyr Thr	Gln Lys Ser Leu Ser Leu	
	650	655	660
	Ser Pro Gly Lys		



664

## (2) INFORMATION FOR SEQ ID NO:19:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 670 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CTGCAAGAAG CTGCGCTCCT CCTACATCTC CATCTGCAAC CGCGAGATCT 50  
10 CGCCCACCGA GCGCTGCAAC CGCCGCAAGT GCCACAAGGC CCTGCGCCAG 100  
TTCTTCGACC GGGTGCCAG CGAGTACACC TACCGCATGC TCTTCTGCTC 150  
CTGCCAAGAC CAGGCGTGCG CTGAGCGCCG CCGGCAAACC ATCCTGCCCCA 200  
GCTGCTCCTA TGAGGACAAG GAGAAGCCCA ACTGCCTGGA CCTGCGTGGC 250  
GTGTGCCGGA CTGACCACCT GTGTCGGTCC CGGCTGGCCG ACTTCCATGC 300  
15 CAATTGTCGA GCCTCCTACC AGACGGTCAC CAGCTGCCCT GCGGACAATT 350  
ACCAGGCGTG TCTGGGCTCT TATGCTGGCA TGATTGGGTT TGACATGACA 400  
CCTAACTATG TGGACTCCAG CCCCCTGGC ATCGTGGTGT CCCCTGGTGC 450  
AGCTGTCGTG GCAGCGGGAA CATGGAGGAG GAGTGTGAGA AGTTCCTCAG 500  
GACTTCACCG AGAACCCATG CCTCCGGAAC GCCATCCAGG CCTTTGGCAA 550  
20 CGGCACGGAC GTGAACGTGT CCCCAAAGG CCCTCGTTCC AGGCCACCCA 600  
GGCCCCTCGG GTGGAGAAGA CGCCTTCTTT GCCAGATGAC CTCAGTGACA 650  
GTACCAGCTT GGGGACCAGT 670

## WHAT IS CLAIMED IS:

1. A polypeptide comprising a sequence selected from the group consisting of:
  - (a) a human NTNR $\alpha$  extracellular domain amino acid sequence;
  - (b) an allelic variant or mammalian homolog of (a);
  - 5 (c) a sequence encoded by nucleic acid which hybridizes under stringent conditions to a nucleic acid encoding (a) or (b);
  - (iv) a sequence derived from (a) or (b) by substitution, deletion, or addition of one or several amino acids in the amino acid sequence of (a) or (b); and,
  - (v) a sequence encoding at least 16 contiguous amino acids from (a), (b) or (c).
- 10 2. The polypeptide of Claim 1, comprising the amino acid sequence of NTNR $\alpha$  ECD from SEQ ID NO: 3 or SEQ ID NO: 6.
3. The polypeptide of Claim 2, comprising the amino acid sequence of mature NTNR $\alpha$  from SEQ ID NO: 3 or SEQ ID NO: 6.
4. The polypeptide of claim 3, which specifically binds neurturin.
- 15 5. The polypeptide of claim 4, which is conjugated with, or fused to, a molecule which increases the serum half-life thereof.
6. The polypeptide of claim 1 that is soluble NTNR $\alpha$ .
7. A composition comprising the polypeptide of claim 1 and a physiologically acceptable carrier.
- 20 8. The NTNR $\alpha$  of claim 1 that is chimeric NTNR $\alpha$ .
9. The chimeric NTNR $\alpha$  of claim 8, comprising a NTNR $\alpha$  amino acid sequence fused to an immunoglobulin sequence.
10. The chimeric NTNR $\alpha$  of claim 8, comprising a NTNR $\alpha$  amino acid sequence fused to an epitope tag.
- 25 11. A method for identifying a molecule which binds to the NTNR $\alpha$ , comprising exposing the NTNR $\alpha$  to the molecule suspected of binding thereto and determining binding of the molecule to the NTNR $\alpha$ .
12. The method of claim 11, wherein the NTNR $\alpha$  is soluble NTNR $\alpha$ .
13. A method for identifying a molecule which activates NTNR $\alpha$ , comprising exposing the NTNR $\alpha$  to a molecule suspected of being capable of activating NTNR $\alpha$  and measuring activation of NTNR $\alpha$ .
- 30 14. A method for purifying a molecule which binds to NTNR $\alpha$ , comprising adsorbing the molecule to NTNR $\alpha$  immobilized on a solid phase and recovering the molecule from the immobilized NTNR $\alpha$ .
15. The method of claim 14 wherein the NTNR $\alpha$  is chimeric NTNR $\alpha$ , comprising a fusion of a NTNR $\alpha$  extracellular domain sequence to an immunoglobulin constant domain sequence.
16. An antibody that specifically binds to NTNR $\alpha$  of claim 1.
- 35 17. The antibody of claim 16, which is a monoclonal antibody.
18. A composition comprising the antibody of claim 17 and a physiologically acceptable carrier.
19. The composition of claim 18 further comprising a cytokine or a neurotrophic factor.
20. A method for activating NTNR $\alpha$  in a cell, comprising exposing the NTNR $\alpha$  to an amount of an agonist antibody of claim 16 which is effective for activating NTNR $\alpha$ .

20. A method for activating NTN $\alpha$  in a cell, comprising exposing the NTN $\alpha$  to an amount of an agonist antibody of claim 16 which is effective for activating NTN $\alpha$ .
21. A method for modulating a physiological response of a cell to NTN, comprising contacting the cell with an amount of a NTN $\alpha$  effective for modulating the response of the cell to NTN.
- 5 22. A method for increasing survival of a Ret-expressing cell, comprising administering to a Ret-expressing cell a survival-promoting effective amount of soluble NTN $\alpha$  or soluble GDNF $\alpha$ .
23. A method for activating Ret on the surface of a cell, comprising administering to the cell a Ret-activating effective amount of soluble NTN $\alpha$  or soluble GDNF $\alpha$ .
24. A method for determining the presence of NTN $\alpha$ , comprising exposing a test sample  
10 suspected of containing the NTN $\alpha$  to the antibody of claim 16 and determining binding of said antibody to the test sample.
25. An isolated nucleic acid molecule comprising a sequence encoding a polypeptide of claim  
1.
26. An isolated nucleic acid molecule comprising a sequence encoding a polypeptide of claim  
15 2.
27. An isolated nucleic acid molecule encoding the polypeptide of claim 8.
28. The isolated nucleic acid molecule of claim 25, further comprising a promoter operably  
linked to the nucleic acid molecule.
29. An expression vector comprising the nucleic acid molecule of claim 25 operably linked to  
20 control sequences recognized by a host cell transformed with the vector.
30. A host cell comprising the vector of claim 29.
31. A process of using a nucleic acid molecule encoding NTN $\alpha$  to effect production of NTN $\alpha$ , comprising culturing the host cell of claim 30 under conditions allowing expression of NTN $\alpha$ .
32. The process of claim 31 further comprising recovering the NTN $\alpha$  from the host cell  
25 culture.
33. A non-human, transgenic animal which contains cells that express nucleic acid comprising the polypeptide of claim 1.
34. The animal of claim 33 which is a mouse.
35. A non-human, transgenic animal which contains cells having an  
30 altered NTN $\alpha$  gene.
36. The animal of claim 35 which is a mouse.



C TAC CGC ATG CTC TTC TGC TCC TGC CAA GAC CAG GCG TGC GCT GAG CGC CGC CAA ACC ATC CTG CCC AGC 857  
 r Tyr Arg Met 220 225 230 235 240 240  
 C TCC TAT GAG GAC AAG GAG AAG CCC AAC TGC CTG GAC CTG GCG ACT GAC CAC CTG TGT CGG 932  
 s Ser Tyr Glu Asp Lys Glu Lys Pro Asn Cys Leu Asp Leu Arg Cys Arg Thr Asp His Leu Cys Arg 265  
 C CGG CTG GCC GAC TTC CAT GCC AAT TGT CGA GCC TCC TAC CAG ACG GTC ACC AGC TGC CCT GCG GAC AAT TAC 1007  
 r Arg Leu Ala Asp Phe His Ala Asn Cys Arg Ala Ser Tyr Gln Thr Val Thr Ser Cys Pro Ala Asp Asn Tyr 290  
 G GCG TGT CTG GGC TCT TAT GCT GGC ATG ATT GGG TTT GAC ATG ACA CCT AAC TAT GTG GAC TCC AGC CCC ACT 1082  
 n Ala Cys Leu Gly Ser Tyr Ala Gly Met 300 305 310 315  
 C ATC GTG GTG TCC CCC TGG TGC AGC TGT CGT GGC AGC GGG AAC ATG GAG GAG GAG TGT GAG AAG TTC CTC AGG 1157  
 y Ile Val Val Ser Pro Trp Cys Ser Cys Arg Gly Ser Gly Asn Met Glu Glu Glu Cys Glu Lys Phe Leu Arg 340  
 C TTC ACC GAG AAC CCA TGC CTC CGG AAC GCC ATC CAG GCC TTT GGC AAC GGC ACG GAC GTG AAC GTG TCC CCA 1232  
 p Phe Thr Glu Asn Pro Cys Leu Arg 345 350 355 360 365  
 A GGC CCC TCG TTC CAG GCC ACC CAG GCC CCT CGG GTG GAG AAG ACG CCT TCT TTG CCA GAT GAC CTC AGT GAC 1307  
 s Gly Pro Ser Phe Gln Ala Thr Gln Ala Pro Arg Val Glu Lys Thr Pro Ser Leu Thr Pro Asp Asp Leu Ser Asp 390  
 T ACC AGC TTG GGG ACC AGT GTC ATC ACC ACC TGC ACG TCT GTC CAG GAG CAG GGG CTG AAG GCC AAC AAC TCC 1382  
 r Thr Ser Leu Gly Thr Ser Val Ile Thr Thr Cys Thr Ser Val Gln Glu Gln Gly Leu Lys Ala Asn Asn Ser 415  
 A GAG TTA AGC ATG TGC TTC ACA GAG CTC ACG ACA AAT ATC ATC CCA GGG AGT AAC AAG GTG ATC AAA CCT AAC 1457  
 s Glu Leu Ser Met Cys Phe Thr Glu Leu Thr Thr Asn Ile Ile Pro Gly Ser Asn Lys Val Ile Lys Pro Asn 440

FIG. 1B

A GGC CCC AGC AGA GCC AGA CCG TCG GCT GCC TTG ACC GTG CTG TCT GTC CTG ATG CTG AAA CTG GCC TTG T 1530  
 r Gly Pro Ser Arg Ala Arg Pro Ser Ala Ala Ala Leu Thr Val Leu Ser Val Leu Met Leu Lys Leu Ala Leu 464  
 445 450 455 460 464  
 GCTGTGGG AACCGAGTCA GAATATTTT GAAAGCTACG CAGACAAGAA CAGCCGCCTG ACGAATGGA AACACACACA GACACACACA CACCTTGCAA 1630  
 AAAAAATT GTTTTCCCA CCTGTGCGCT GAACCTGTCT CCTCCCAGGT TTCTTCTCTG GAGAAGTTTT TGTAAACCAA ACAGACAAGC AGGCAGGCAG 1730  
 TGAGAGCT GGCCCAAGGG TCCCCTGGCA GGGGAAACTC TGGTGCCGGG GAGGGCACGA GGCTCTAGAA ATGCCCTTCA CTTTCTCCTG GTGTTTTTCT 1830  
 CTGGACCC TTCTGAAGCA GAGACCGGAC AAGAGCCTGC AGCGGAAGGG ACTCTGGGT GTGCCTGAGG CTGGCTGGGG GCAGGACAAC ACAGCTGCTT 1930  
 CCAGGCTG CCCACTCTGG GGACCCGCTG GGGGCTGGCA GAGGCATCG GTCAGCGGG CAGCGGGCT GGCCATGAGG GTCCACCTTC AGCCCTTTGG 2030  
 TCAAGGAT GGAGATGGTT TTGCCCTCCC TCTCTGCCCT CGGGTGGGC TGGTGGTCT GCAGCTGGTG TGGGAACTTC CCCACGGATG GCGGTGGAGG 2130  
 GTTCGCAC CGTGCTGGG TCCCCCTGAC TGTAGCACGG AGTGTGGG CTGGGGGCCA GCTCCAGGAG GGCTTGAGAG CTCAGCCTGC CTGGGAGAGC 2230  
 TTGTGGCG AGGCATTAA ACTTGGGCAC CAGCTTCTTT CTCGGTGGCA GAAATTTGA AGTCAGAGAG AAACGGTCTT TTGTTGGCTT CTTTGCTTTC 2330  
 GTGGGTCC TTTGGCAGGC CTCCCTTTGG GGAGAGAGACC ACAGCCGGGT GTGTGTCTGC AGCACCGTGG GCCCTCAAGC TTTCCCTGCTG 2430  
 TTCTCCCT CCTCCTCCTT TCCCCTTCT CTTTCTCTCAT TTCCTAGACG TACGTCAACT GTATGTACAT ACCGGGGCTC CTCCTCTAAC ATATATGTAT 2530  
 ACACATCC ATATACATAT ATTGTGTGGT TTCCCCTTTC TTTCTTTTTT TAAGCAACAA AACTATGGGG 2600

FIG. 1C

100 TGGCGG CCGCTCTAGA ACTAGTGGAT CCCCGGGCTG CAGGAATTGG GCACGAGAGT GAGCCGAGCA AGGTTAGCG GGAGAAGATT TTTTCTTTT  
 200 TCTTTT TCTTGTCTT GGTGCCAAG AAGCGACTCT GGTCTCCCGT CCTAGAAGCT CCTACTGGAT TGCTCCTATT CCGTCGGTGG ATTTCTTTCC  
 300 CGCATT TATTCTGACC CCTCCCTCG CTGCTTCTTT CCAGCCCTTC ACTTTCAGAT CGCTCGGCC CCACCTCTCC AGTCCCCTCC TGGGAAGTGC  
 400 GAAATTG GACCCACGGG GACTCAGCC TTCCCGGACG CGCGAGCAA GGGCTGGCT GACCTCAGGA CCAGGCTGTT GGCTTAGAAG GCAGCCAGAC  
 500 TAGTAC GTGTGTTTGA TTTCAAGTGC AAGGGGGAC GTCGAGAGGC AGCCACCGC CGCCTCCTA CCCCTCCCC TCCAACCAGC AGTGAGAATC  
 600 GACTCG GGATCTTCAA CCGGGGGCG CCGGGGGGA TCTCCGCAAT GGATTGGGG GTCGTTATTG CTCGGCTGTT ATTATTATCG TTATTTTATT  
 688 TTTTAAACCTAAGG GAGAAAGACA CATAACACA AAACGTGGG ATTTATTAA C ATG ATC TTG GCA AAC GCC TTC TGC CTC TTC  
 Met Ile Leu Ala Asn Ala Phe Cys Leu Phe  
 1 5 10  
 766 TTT TTA GAC GAA ACC CTC CGC TCT TTG GCC AGC CCT TCC TCC CTG CAG GGC TCT GAG CTC CAC GGC TGG CGC  
 Phe Leu Asp Glu Thr Leu Arg Ser Leu Ala Ser Pro Ser Ser Leu Gln Gly Ser Glu Leu His Gly Trp Arg  
 15 20 25 30 35  
 841 CAA GTG GAC TGT GTC CGG GCC AAT GAG CTG TGT GCG GCT GAA TCC AAC TGC AGC TCC AGG TAC CGC ACC CTT  
 Gln Val Asp Cys Val Arg Ala Asn Glu Leu Cys Ala Ala Glu Ser Asn Cys Ser Ser Arg Tyr Arg Thr Leu  
 40 45 50 55 60  
 916 CAG TGC CTG GCA GGC CGG GAT CGC AAT ACC ATG CTG GCC AAT AAG GAG TGC CAG GCA GCC CTG GAG GTC TTG  
 Gln Cys Leu Ala Gly Arg Asp Arg Asn Thr Met Leu Ala Asn Lys Glu Cys Gln Ala Ala Leu Glu Val Leu  
 65 70 75 80 85  
 991 GAA AGC CCA CTG TAT GAC TGC CGC TGC AAG CGG GGC ATG AAG AAG GAG CTG CAG TGT CTG CAG ATC TAC TGG  
 Glu Ser Pro Leu Tyr Asp Cys Arg Cys Lys Arg Gly Met Lys Lys Glu Leu Gln Cys Leu Gln Ile Tyr Trp  
 90 95 100 105 110  
 1066 ATC CAT CTG GGG CTG ACA GAG GGT GAG GAG TTC TAT GAA GCT TCC CCC TAT GAG CCT GTG ACC TCG CGC CTC  
 Ile His Leu Gly Leu Thr Glu Gly Glu Glu Phe Tyr Glu Ala Ser Pro Tyr Glu Pro Val Thr Ser Arg Leu  
 115 120 125 130 135

FIG. 2A

3 GAC ATC TTC AGG CTC GCT TCA ATC TTC TCA GGG ACA GGG ACA GAC CCG GCG GTC AGT ACC AAA AGC AAC CAC 1141  
 r Asp Ile Phe Arg Leu Ala Ser Ile Phe Ser Gly Thr Gly Thr Asp Pro Ala Val Ser Thr Lys Ser Asn His 160  
 140  
 3 CTG GAT GCC GCC AAG GCC TGC AAC CTG AAT GAC AAC TGC AAG AAG CTT CGC TCC TCT TAT ATC TCC ATC TGC 1216  
 s Leu Asp Ala Ala Lys Ala Cys Asn Leu Asn Asp Asn Cys Lys Lys Leu Arg Ser Ser Tyr Ile Ser Ile Cys 185  
 165 170 175 180 185  
 C CGT GAG ATC TCT CCC ACC GAA CGC TGC AAC CGC AAG TGC CAC AAG GCT CTG CGC CAG TTC TTT GAC CGT 1291  
 n Arg Glu Ile Ser Pro Thr Glu Arg Cys Asn Arg Arg Lys Cys His Lys Ala Leu Arg Gln Phe Phe Asp Arg 210  
 190 195 200 205 210  
 3 CCC AGC GAG TAT ACC TAC CGC ATG CTC TTC TGC TCC TGT CAG GAC CAG GCA TGT GCT GAG CGT CGC CGG CAA 1366  
 1 Pro Ser Glu Tyr Thr Tyr Arg Met Leu Phe Cys Ser Cys Gln Asp Gln Ala Cys Ala Glu Arg Arg Gln 235  
 215 220 225 230 235  
 C ATC CTG CCC AGT TGC TCC TAT GAG GAC AAG GAG AAG CCC AAC TGC CTG GAC CTG CGC AGC CTG TGT CGT ACA 1441  
 r Ile Leu Pro Ser Cys Ser Tyr Glu Asp Lys Glu Lys Pro Asn Cys Leu Asp Leu Arg Ser Leu Cys Arg Thr 260  
 240 245 250 255 260  
 C CAC CTG TGC CGG TCC CGA CTG GCA GAT TTC CAC GCC AAC TGT CGA GCC TCC TAC CGG ACA ATC ACC AGC TGT 1516  
 p His Leu Cys Arg Ser Arg Leu Ala Asp Phe His Ala Asn Cys Arg Ala Ser Tyr Arg Thr Ile Thr Ser Cys 285  
 265 270 275 280 285  
 T GCG GAC AAC TAC CAG GCA TGT CTG GGC TCC TAT GCT GGC ATG ATT GGG TTT GAT ATG ACA CCC AAC TAT GTG 1591  
 o Ala Asp Asn Tyr Gln Ala Cys Leu Gly Ser Tyr Ala Gly Met Ile Gly Phe Asp Met Thr Pro Asn Tyr Val 310  
 290 295 300 305 310

FIG. 2B



1666  
 1741  
 1816  
 1891  
 1966  
 2041

TCC AAC CCC ACG GGC ATC GTG GTG TCT CCC TGG TGC AAT TGT CGT GGC AGT GGC AAC ATG GAA GAA GAG TGT  
 Ser Asn Pro Thr Thr Gly Ile Val Val Ser Pro Trp Cys Asn Cys Arg Gly Ser Gly Asn Met Glu Glu Cys  
 315 320 325 330 335

AAG TTC CTC AGG GAC TTC ACG GAA AAC CCA TGC CTC CGG AAT GCC ATT CAG GCC TTT GGT AAT GGC ACA GAT  
 Lys Phe Leu Arg Asp Phe Thr Glu Asn Pro Cys Leu Arg Asn Ala Ile Gln Ala Phe Gly Asn Gly Thr Asp  
 340 345 350 355 360

AAC ATG TCT CCC AAA GGC CCC TCA CTC CCA GCT ACC CAG GCC CCT CGG GTG GAG AAG ACT CCT TCA CTG CCA  
 Asn Met Ser Pro Lys Gly Pro Ser Leu Pro Ala Thr Gln Ala Pro Arg Val Glu Lys Thr Pro Ser Leu Pro  
 365 370 375 380 385

GAC CTC AGT GAC AGC ACC AGC CTG GGC ACC AGT GTC ATC ACC ACC TGC ACA TCT ATC CAG GAG CAA GGG CTG  
 Asp Leu Ser Asp Ser Thr Ser Leu Gly Thr Ser Val Ile Thr Thr Cys Thr Ser Ile Gln Glu Gln Gly Leu  
 390 395 400 405 410

GCC AAC AAC TCC AAA GAG TTA AGC ATG TGC TTC ACA GAG CTC ACG ACA AAC ATC AGT CCA GGG AGT AAA AAG  
 Ala Asn Asn Ser Lys Glu Leu Ser Met Cys Phe Thr Thr Thr Thr Asn Ile Ser Pro Gly Ser Lys Lys  
 415 420 425 430 435

ATC AAA CTT AAC TCA GGC TCC AGC AGA GCC AGA CTG TCG GCT GCC TTG ACT GCC CTC CCA CTC CTG ATG CTG  
 Ile Lys Leu Asn Ser Gly Ser Ser Arg Ala Arg Leu Ser Ala Ala Leu Thr Ala Leu Pro Leu Leu Met Leu  
 440 445 450 455 460

FIG. 2C

: TTG GCC TTG T AGGCCT TTGGAACCCA GCACAAAAGT TCTTCAAGCA ACCCAGATAT GAACTCCGC CTGACAAAAT GGAAACACAC GCATACACAC 2140  
 : Leu Ala Leu 464  
 CACACAC ACACAAACAC ACACACACAC ACACACACAC ACACACACAC ACACCCCTTG CAAAAACACT TTTTTCCTA CATGTCTCT 2240  
 CCTTTCT CCTCCCAAGT TTCTTCTCTG GAGAAGTTT TCTAAACCAA ACAGACAAGC AGGCGGCAG TCAGAAGCCT GCCCAGAGGT CCCCTGCAAG 2340  
 CACCCAG CACCAACGAG GGCTCAAGC TCTTGAGAGA CTCCTTTCTC TTCACTGGTG TTTTCTCTCT GGACAAGATG AGACCCCTGAT GTGGAAGGTA 2440  
 TGCTGTG CTGGGTGTGG ACTGGGGAAA GGACAGTTGC AGCTGCCTAC TCTGGGACC TGCCCAAGGG TTCACAGAGA GTCTCAGTCA GCAAGGAAGC 2540  
 GCTGGCC ACAAGGACTT TGTCACTCT TCCCTCTTGGC TTCAGAGATG GAAATGGTTT GCTGCCATCC CCAGCCATTA TGTGGCCTAG TGGGTTTAAG 2640  
 GGAGTAG GAAGCCTCAT GGCAGCTTCA GGCCATGGTG CCTGTAGTAT AGCTGGGGTT GGGAGCTGTT ACAGGAGGAA GCTTCTTTGG GGCATGAGCA 2740  
 CTTGGTT GGGCACCAGC TCCAAGATGT ACCTTCCTCC TTATATGCCAG GAATCTTGAA GTCAAAAGAGA AATGATCCTC TGTGGGCTCT TTTTGTGTTT 2840  
 TTGAATT TTTTGTGGG TCCATTGGC AGGTCTCTCT TGGGAGAAAG GGCTGTTGAG TGGGGCTGGG GAGACCCCTAG CTGGGCGTGT GTATGGAGCA 2940  
 TGGTGGG TTCCCAAGCT TGCCCCCTCT CTCCTCTTGT TTCTGCTTTC TCTCTCAATT CTGAGACATT CATGCACTGT CTGTACATAC TGGGTCTCCT 3040  
 TCAACAT ATGTGTATAT CCATATCCAT ATATCCTATG ATTTACTCT TTCTTTCAAT TTTTAAAG AAACAAAACAT ATGGAAATAA TACCCTACAG 3140  
 AGCGAA ATGTATTATT GTAAAGTTTA TTTTTTTTAA TAATGTTGTC TATGATGGGA AGAAAGGTAC CAGGACCCCT GAGCCCTGGT CCAGTTGGGC 3240  
 TGGGGCT GTGGCCGGGG ACTCCCGATT GCATTCACTC TTAACCAAGC TCCAATAAAC GTACTAGGAA GCGAAAAAAA AAAAAAAA ACTCGAGGGG 3340  
 CCGGGTA CCCAATTC 3358

FIG. 2D

	1	A T G A T C T T G G C A A A C G T	C T T C T T	C C T C T T C T T C T T	T C T A G A C G A	G A C C C T
rAlpha.coding						
fAlpha.coding	1	- - - - -	- - - - -	- - - - -	A T G T T C C T A G C	A C T C T
rAlpha.coding	1	A T G A T C T T G G C A A A C G C	C T T C T T	G C C T C T T C T T C T T T	T T A G A C G A	A C C C T
rAlpha.coding	51	C C G C T C T T T G G C C A G C C C T	T C C T C C C T G C A G A	C C C G A G C T C C A C G G C T		
fAlpha.coding	18	G T A C T T C G C G	C T G C C A C T C C T G G A T T	T G C T G A T G T C C G C C G A G G T G A	G T G	
rAlpha.coding	51	C C G C T C T T T G G C C A G C C C T	T C C T C C C T G C A G G	C T C T G A G C T C C A C G G C T		
rAlpha.coding	101	G G C G C C C C C	C A G T G G A C T G T G T C C G G G C C A A T	G A G C T G T G T G C T G C G A A		
fAlpha.coding	68	G T G G A G A C C G T C T	G G A C T G T G T G A A A G C C A G C G A T C A	G T G C C T G A A G G A A		
rAlpha.coding	101	G G C G C C C C C A	A G T G G A C T G T G T C C G G G C C A A T	G A G C T G T G T G C G G C T G A A A		
rAlpha.coding	151	T C C A A C T G C A G C T C T C G C T	A C C G C A C T	C T G C G G C A G T G C C T G G C A G G C C G		
fAlpha.coding	118	C A G A G C T G C A G C A C C A A	G T A C C G C A C A C T A A	G G C A G T G C G T G G C G G G C A A		
rAlpha.coding	151	T C C A A C T G C A G C T C C A G G T A C C G C A C C C T	T C C G C A G T G C C T G G C A G G C C G			
rAlpha.coding	201	C G A C C G C A A C A C C A T G C T G	- - - - -	- - - - -	G C C A A C A A G G A G T G C C	
fAlpha.coding	168	G G A A A C C A A C T T C A G C C T G A C A T C C G G C C T T G A G	G C C C A A G G C C A A G G A T G A G T G C C			
rAlpha.coding	201	G G A T C G C A A T A C C A T G C T G	- - - - -	- - - - -	G C C A A T A A G G A G T G C C	
rAlpha.coding	236	A G G C G C C C T T G G A G G T C T T G C A G G A G A G C C C G				
fAlpha.coding	218	G T A G C G C C A T G G A G G C C T T G A G C A G A A G T C T	G T G T A C A A C T G C C G C T G C			
rAlpha.coding	236	A G G C A G C C C T G G A G G T C T T G C A G G A A A G C C C A	C T G T A T G A C T G C C G C T G C			

**FIG. 3A**

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NRalpha.coding	286	AAGCGGGGCATGAAGAAAGGAGCTGCAGTGTCTCTGCAGATCTACTGGAGCAT
NFRalpha.coding	288	AAGCGGGGCATGAAGAAAGGAGCTGCAGTGTCTCTGCAGTATCTACTGGAGCAT
NRalpha.coding	286	AAGCGGGGCATGAAGAAAGGAGCTGCAGTGTCTCTGCAGATCTACTGGAGCAT
NRalpha.coding	336	CCACCTGGGGCTGACCGAGGGGTGAGGAGTTCTACGGAAGCCCTCCCCCTATG
NFRalpha.coding	318	GTACCAAGAGCCTGC--AGGGAAATGACCTCTCTGGAAGATTTCCCCCGTATG
NRalpha.coding	336	CCATCTGGGGCTGACAGAGGGGTGAGGAGTTCTATGGAAGCTTTCCCCCTATG
NRalpha.coding	386	AGCCGGTGACCTCCCGCCTCTCGGACATCTTCAAGGCTTGCTTCAATCTTC
NFRalpha.coding	385	AGCCGGTTACAGCAGGTTGTCAAGTATATTCCTGGGCAGTCCCGCTTCAATA
NRalpha.coding	386	AGCCCTGTGACCTCGCGCCTCTCGGACATCTTCAAGGCTTGCTTCAATCTTC
NRalpha.coding	436	TCAAGGACAGGGGTCAGACCCGGTGGTCAGCGCCAGAGCAGCAACCAATTGCCCT
NFRalpha.coding	415	TCAGATGTTTCCAGCAAGTGGACACATTTCCAAAGGAGCAACTGCCCT
NRalpha.coding	436	TCAAGGACAGGGGACAGACCCGGCGGTCAGTACCAAAAGCAACCACTGCCCT
NRalpha.coding	486	GGATGGTGCCAAAGGCCCTGCAACCTGAATGACAACTGCAAGAGCTGCGCT
NFRalpha.coding	465	GGACGGCAGCCAAAGGCCCTGCAACCTGAGACACCTGTAAAGAGTACAGGT
NRalpha.coding	486	GGATGGCGCCAAAGGCCCTGCAACCTGAATGACAACTGCAAGAGCTTGCGCT

FIG. 3B

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Ralpha.coding	536	CCTCCTACATCTCCATCTGCAACCGCGAGATCTCGCCACCGAGCGGCTGC
FRalpha.coding	515	C[G]G[C]CTACATCA[C]C[C]CTGCA[C]C[C]C[C]A[C]GAGTCTGC
Ralpha.coding	536	CCTCTTATATCTCCATCTGCAACCGTGAGATCTCTCCGACCGAACGCTGC
Ralpha.coding	586	AACCGCGCAAGTGCCACAAGGCCCTGCGCCAGTTCTTCGACCGGTGCC
FRalpha.coding	562	AACCGCGCGTAAAGTGCCACAAGGCCCTCAGGCAAGTTCTTCGACCGTGTGCC
Ralpha.coding	586	AACCGCGCAAGTGCCACAAGGCTCTGCGCCAGTTCTTCGACCGTGTGCC
Ralpha.coding	636	CAGCGAGTACACCTACCGCATGCTCTTCTGCTCCTGCCAAGACCAAGGCT
FRalpha.coding	612	GGC[A]AGCACA[G]CTAC[G]GATGCTCTTCTGCTCCTGCCGATCGGCGT
Ralpha.coding	636	CAGCGAGTATACCTACCGCATGCTCTTCTGCTCCTGTCAAGGACCAAGGCA
Ralpha.coding	686	GCGCTGAGCGGC
FRalpha.coding	662	GCACCGAGCGGC
Ralpha.coding	686	GTGCTGAGCGGT
Ralpha.coding	686	CGCCGGGCAACCATCTGCTGCCAGC
FRalpha.coding	662	CGCGAGCACTATCGTCCCGTG
Ralpha.coding	686	CGCCGGGCAACCATCTGCTGCCAGT
Ralpha.coding	686	TGCTCCTATGAGGAC
FRalpha.coding	662	TGCTCCTATGAGAA
Ralpha.coding	686	TGCTCCTATGAGGAC

FIG. 3C

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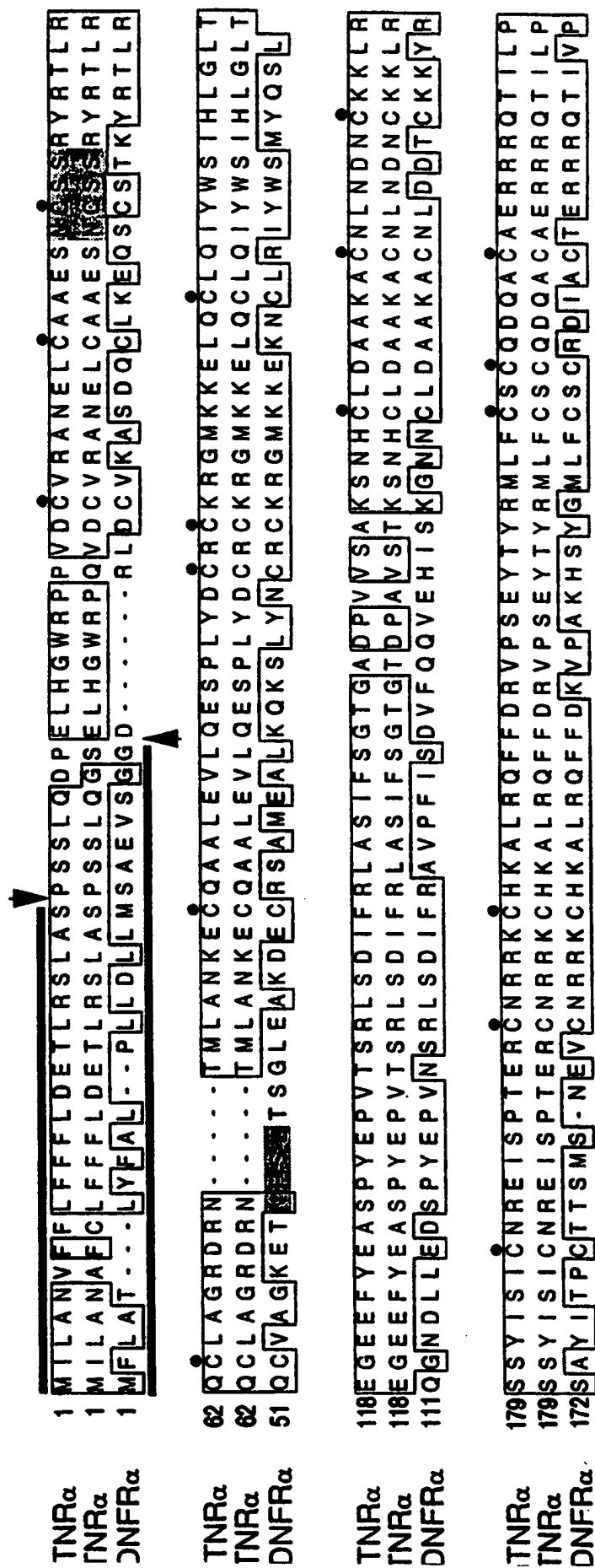


FIG. 4A

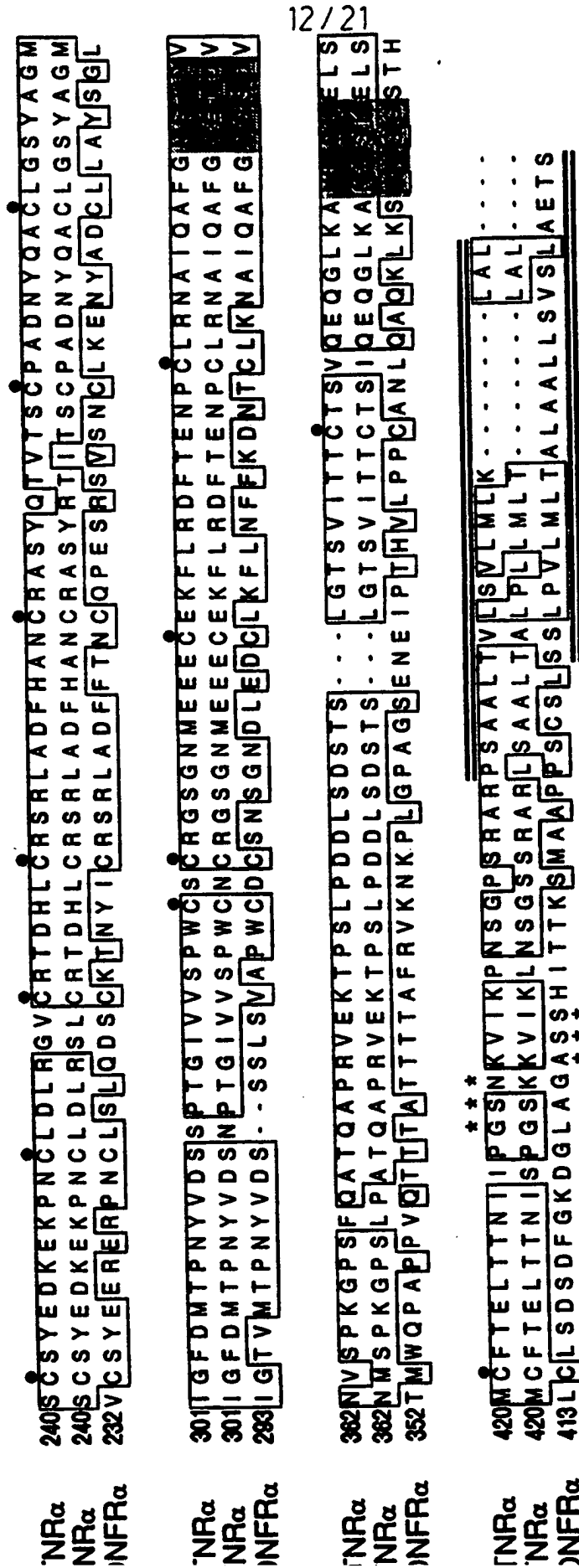


FIG. 4B

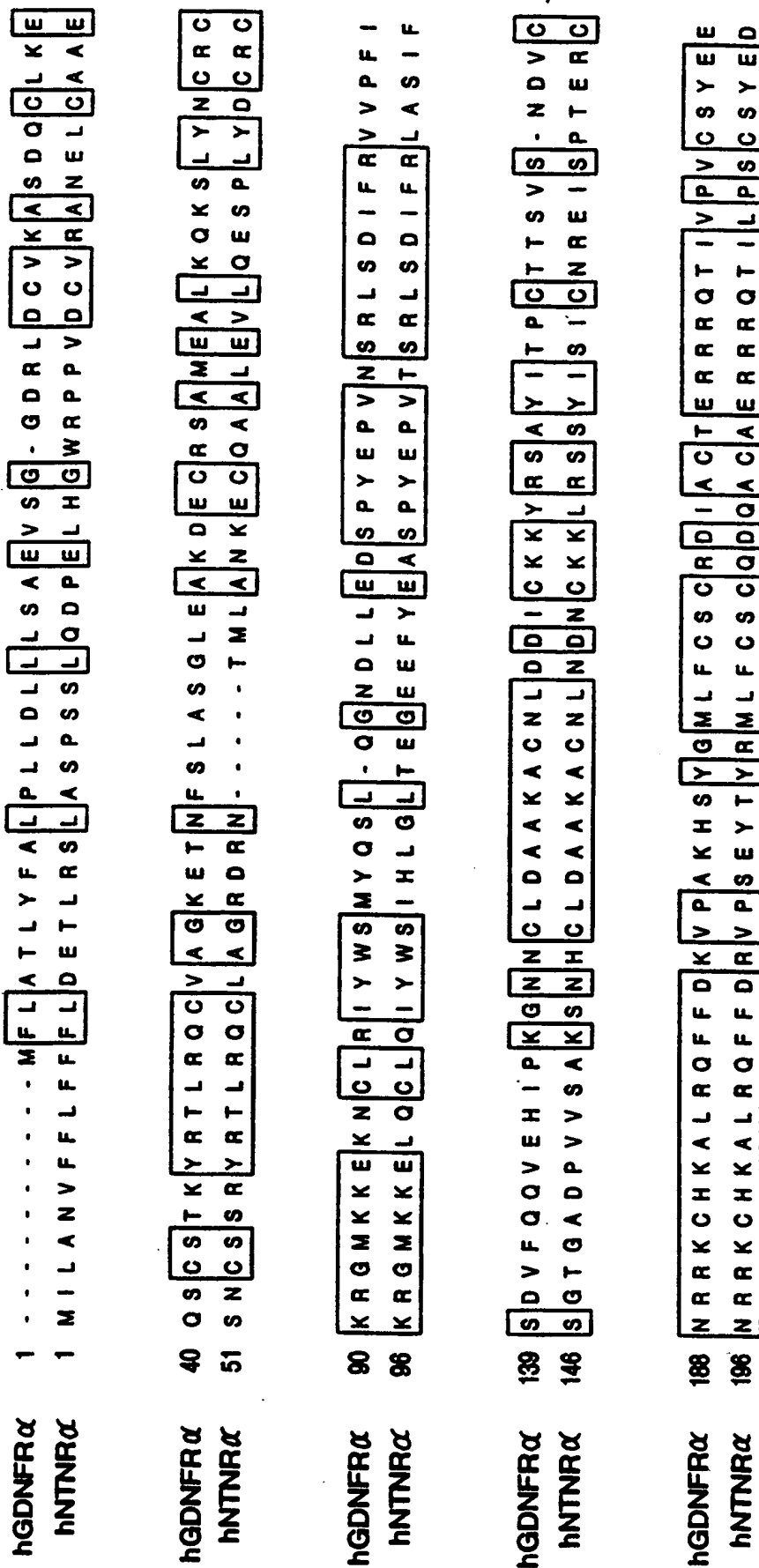


FIG. 5A



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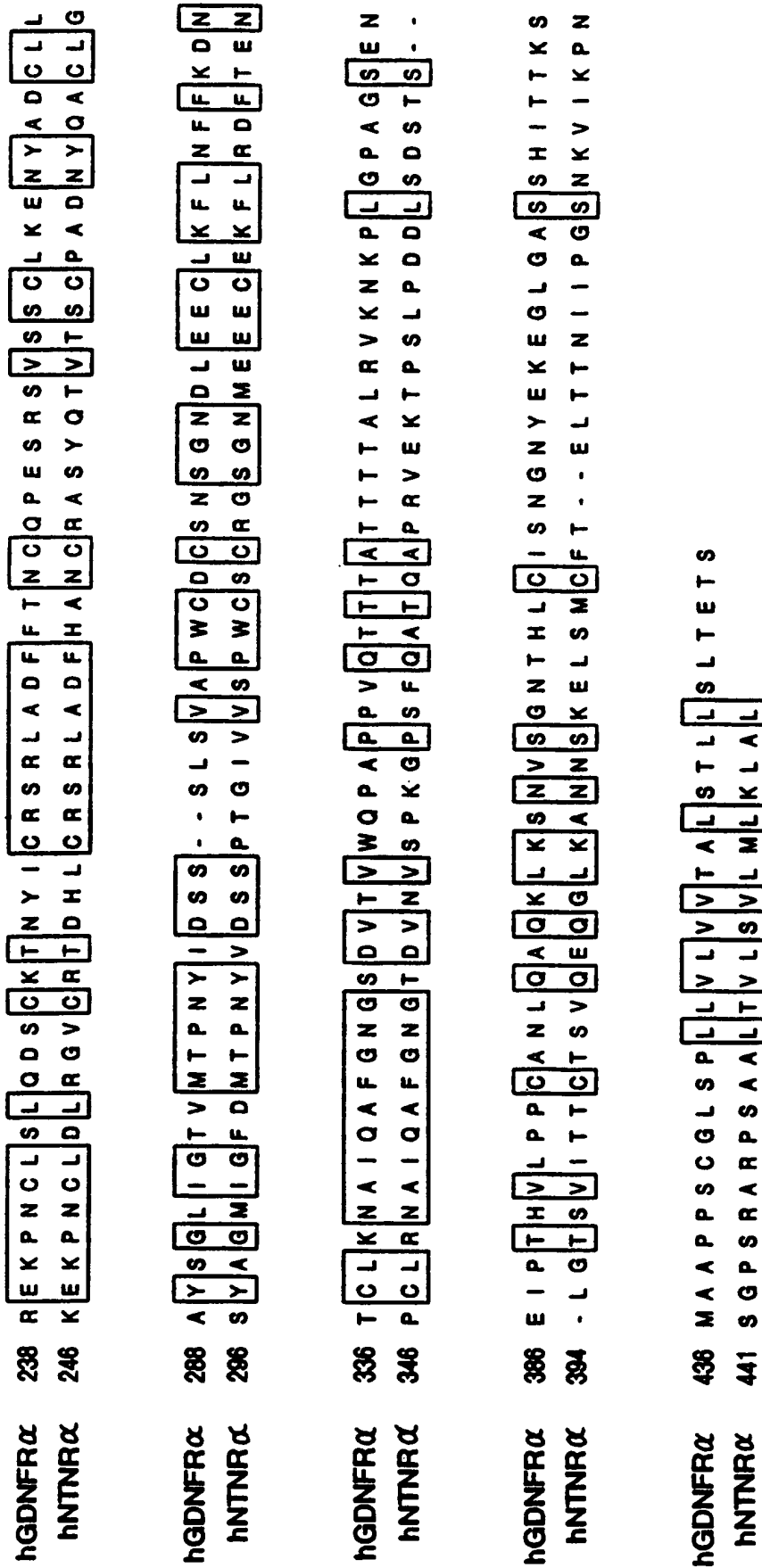


FIG. 5B

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○ mNTN

● rGDNF

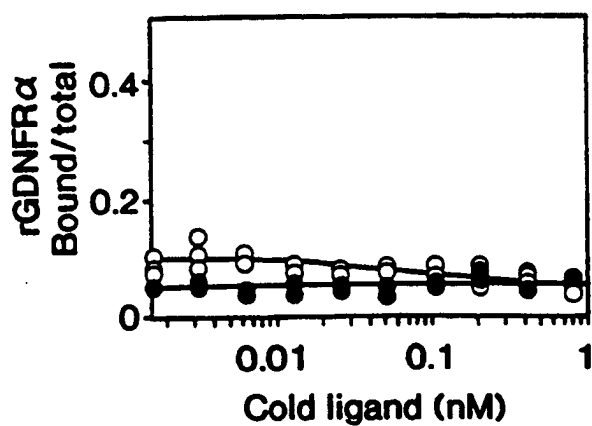


FIG. 6A

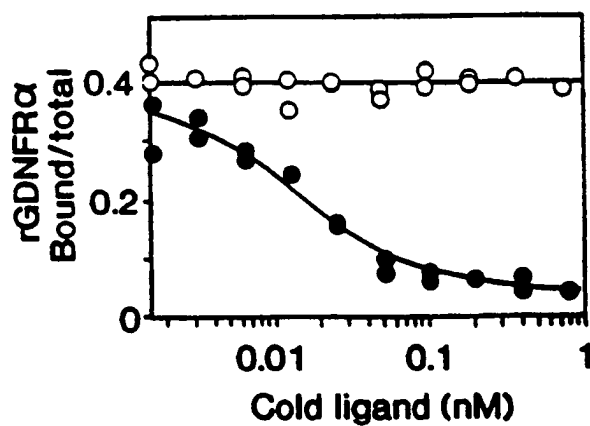


FIG. 6B

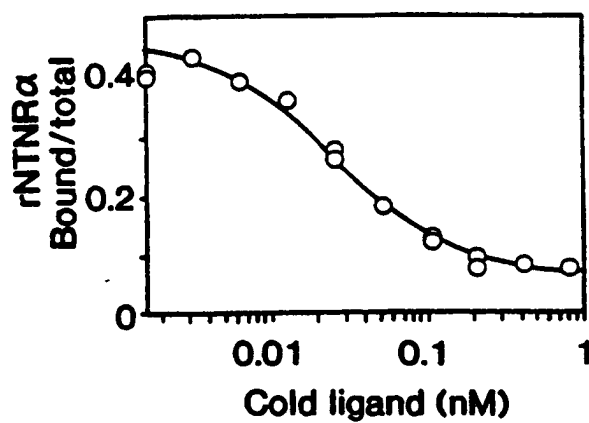


FIG. 6C

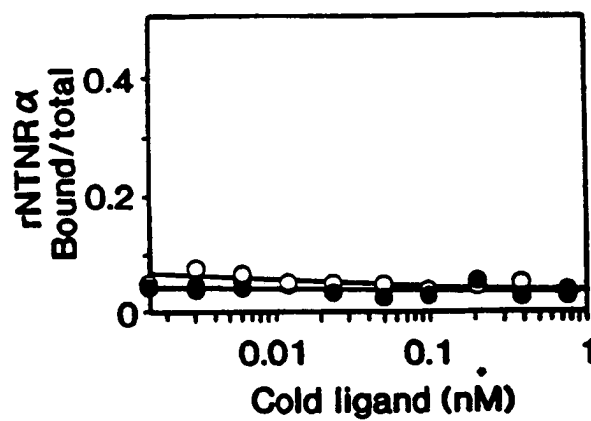


FIG. 6D

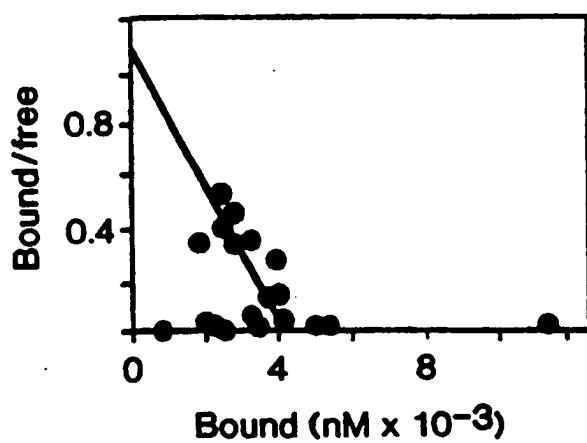


FIG. 6E

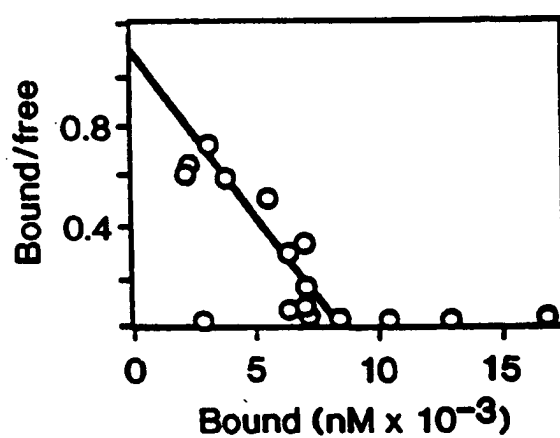


FIG. 6F

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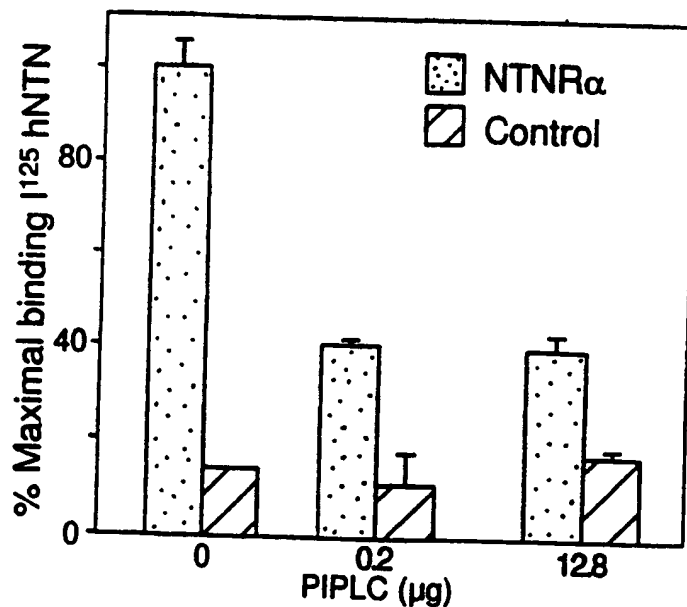


FIG. 7A

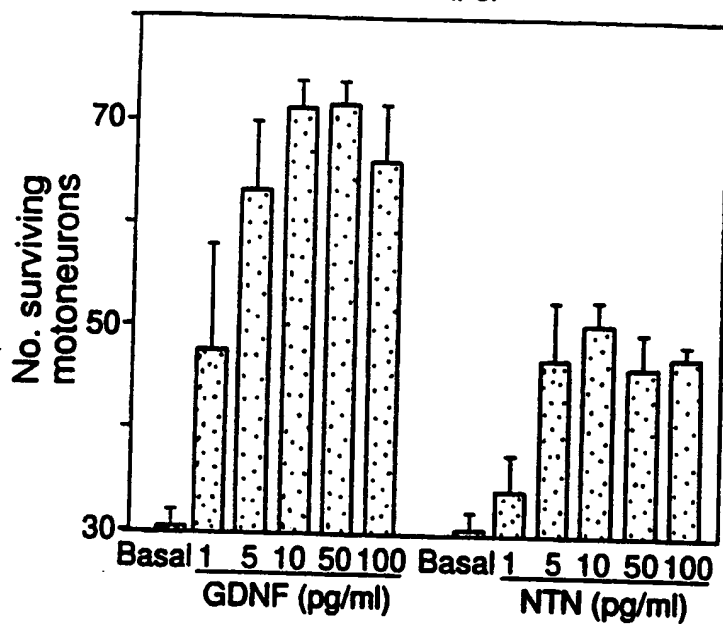


FIG. 7B

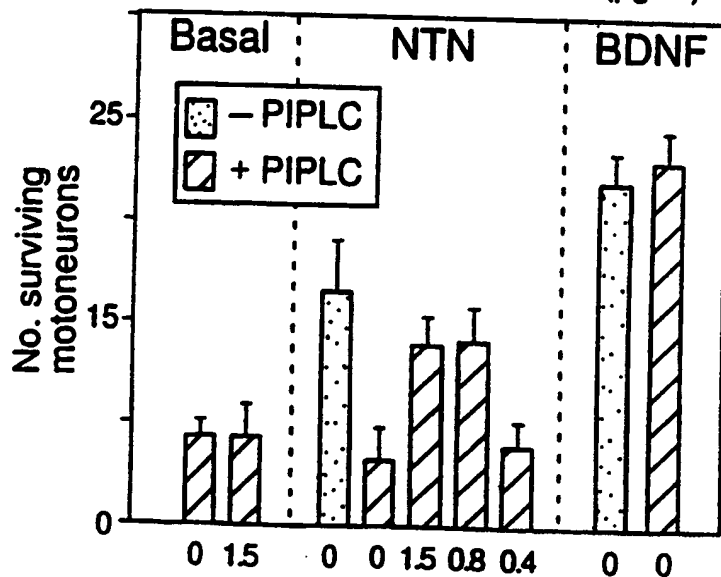


FIG. 7C

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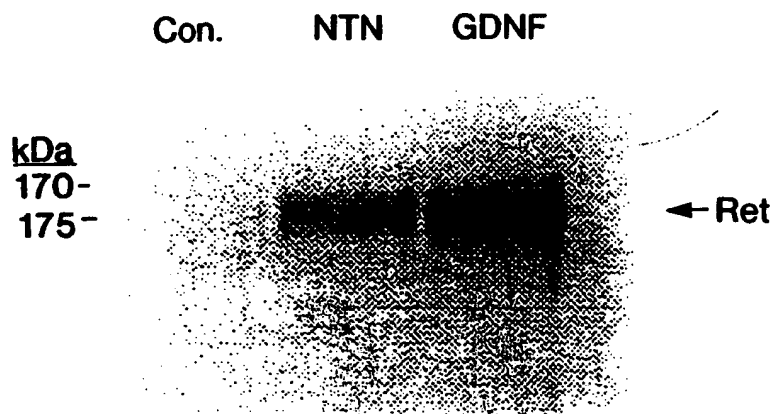


FIG. 7D

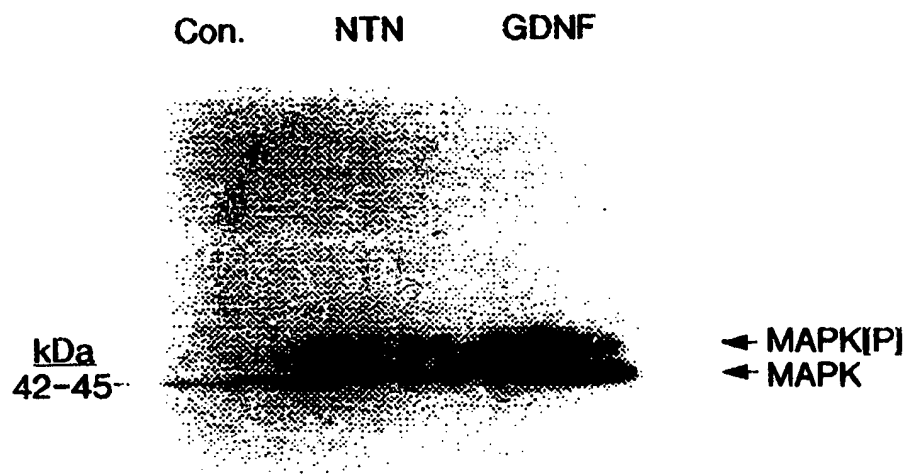


FIG. 7E

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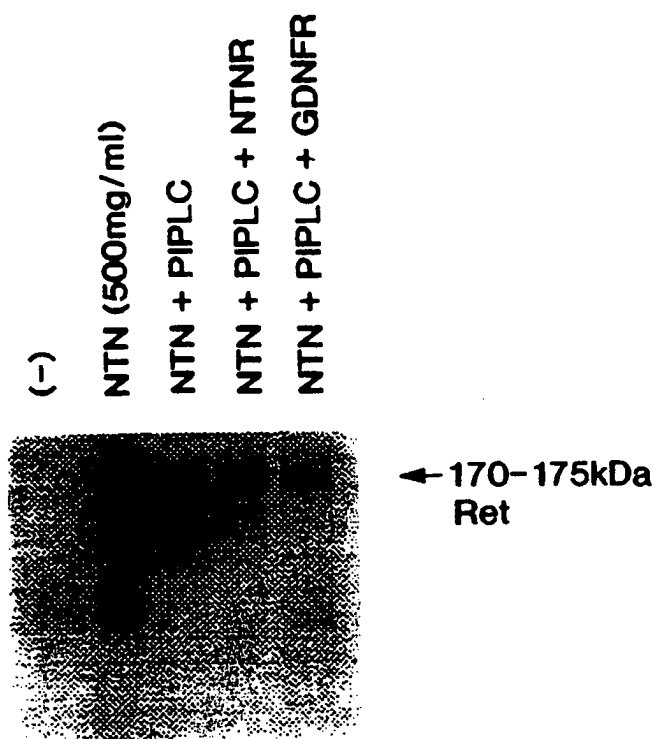


FIG. 7F

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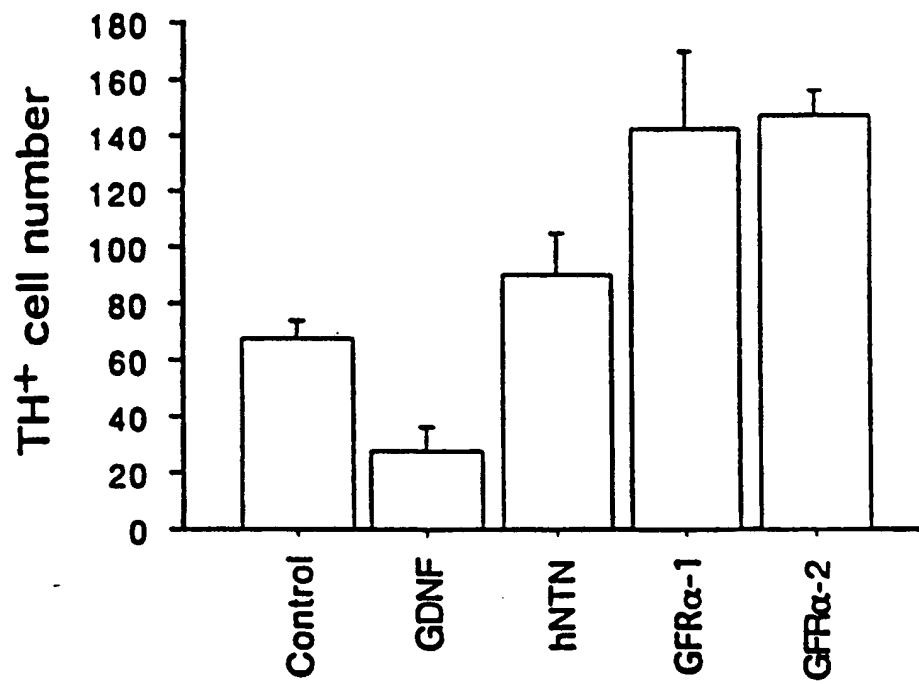


FIG. 8A

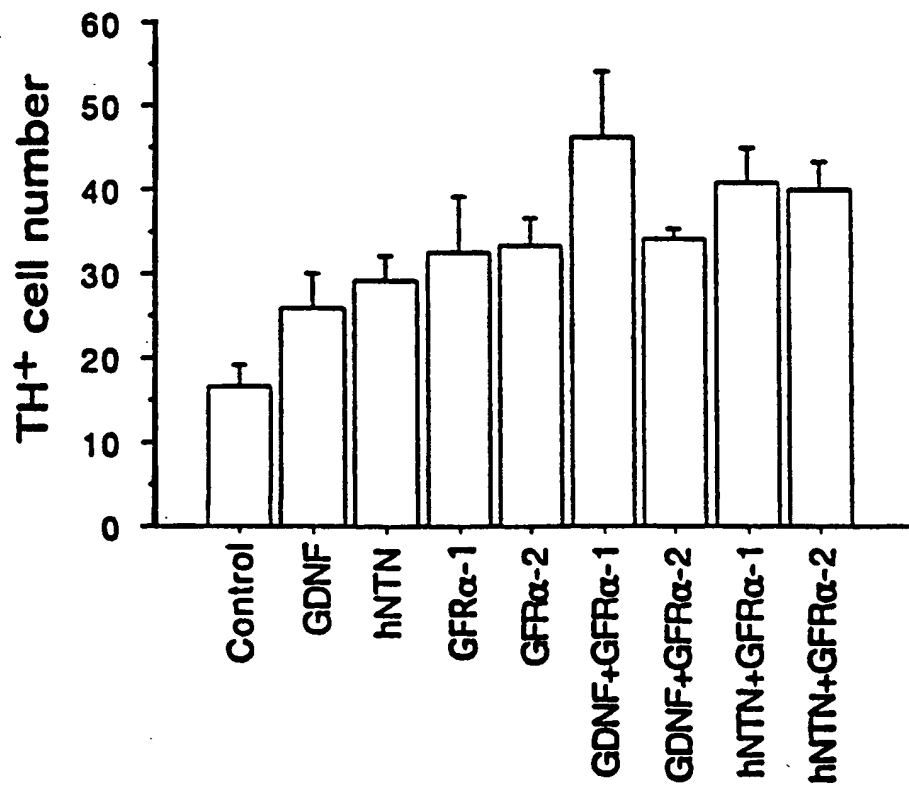


FIG. 8B

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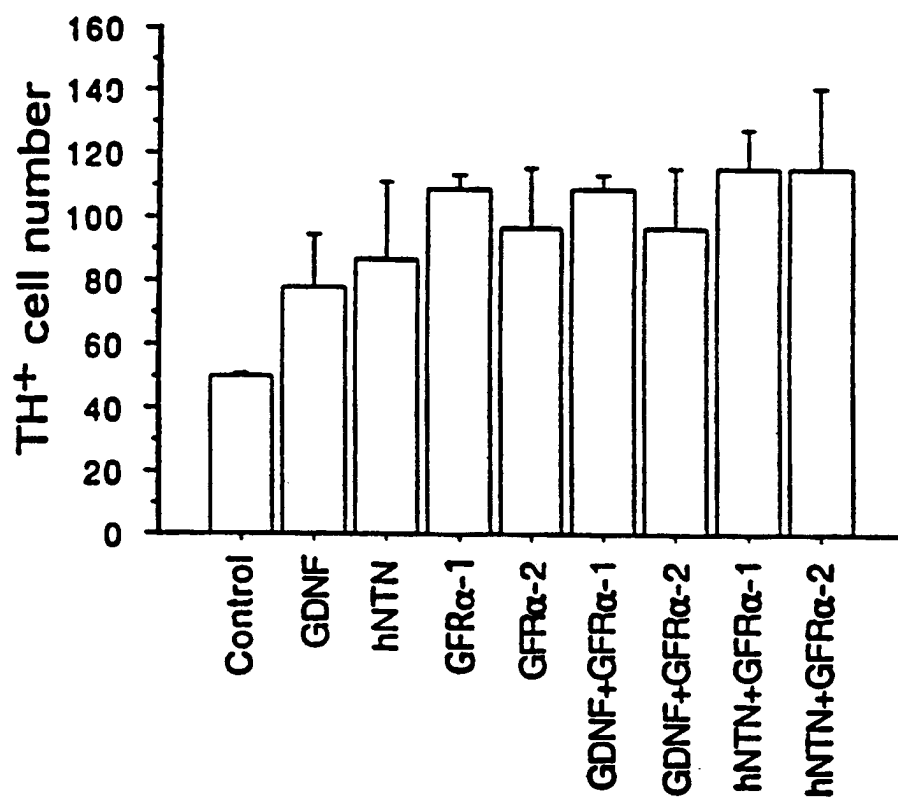
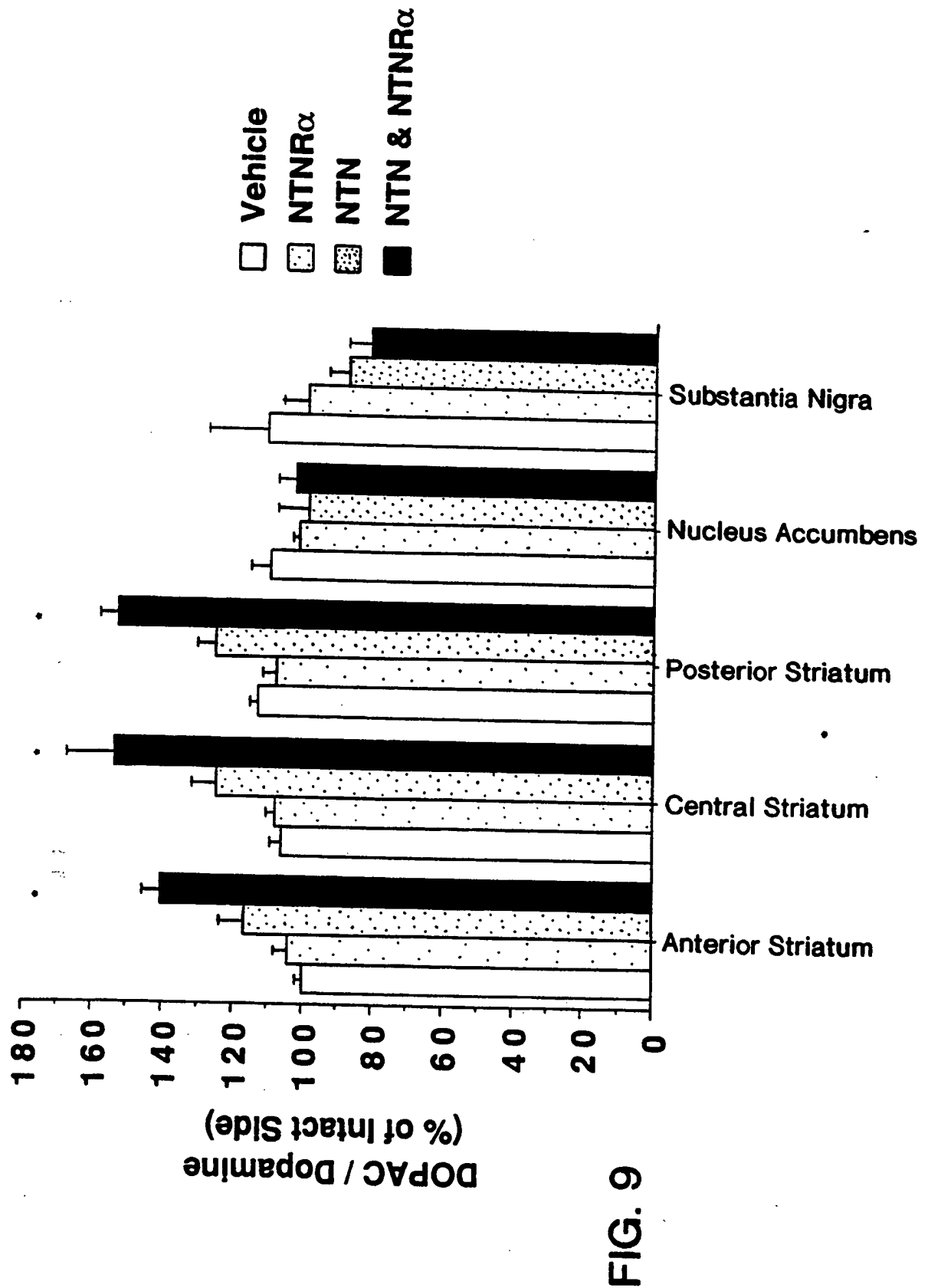


FIG. 8C

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/03179

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/71 C07K19/00 C07K16/46 G01N33/68  
 C07K1/22 C07K16/28 A61K38/17 A61K39/395 C12N15/85  
 C12N5/10 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHUQIAN JING ET AL.: "GDNF-induced activation of the Ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF" CELL, vol. 85, no. 7, 28 June 1996, NA US, pages 1113-1124, XP002065819 cited in the application	22,23
A	see the summary see page 1114, right-hand column, last paragraph - page 1120, right-hand column, paragraph 2 <div style="text-align: center;">---</div> <div style="text-align: center;">-/--</div>	1-21, 24-37

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

Date of the actual completion of the international search

26 May 1998

Date of mailing of the international search report

03.07.98

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Authorized officer

Montero Lopez, B

## INTERNATIONAL SEARCH REPORT

Intern: at Application No

PCT/US 98/03179

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>KLEIN-R D ET AL: "A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor [published erratum appears in Nature 1998 Mar 12;392(6672):210]."</p> <p>NATURE, vol. 387, no. 6634, 12 June 1997, LONDON GB, pages 717-721, XP002065820 see the whole document</p> <p>---</p>	1-37
P,X	<p>WO 97 44356 A (BIOGEN, INC.) 27 November 1997</p> <p>see page 2, line 10 - line 19 see page 2, line 24 - page 4, line 15 see page 29, line 8 - page 31, line 12 see page 34, line 19 - page 43, line 2; figure 7</p> <p>---</p>	1-10, 16-37
P,X	<p>BALOH R H ET AL: "TrnR2, a novel receptor that mediates neurturin and GDNF signaling through Ret."</p> <p>NEURON, vol. 18, no. 5, May 1997, pages 793-802, XP002065821 see the summary see page 793, right-hand column, paragraph 4 - page 794, right-hand column, paragraph 2 see page 797, right-hand column, paragraph 2 - page 800, left-hand column, paragraph 3; figure 1</p> <p>---</p>	1-37
P,X	<p>BUJ-BELLO A ET AL: "Neurturin responsiveness requires a GPI-linked receptor and the Ret receptor tyrosine kinase."</p> <p>NATURE, vol. 387, no. 6634, 12 June 1997, LONDON GB, pages 721-724, XP002065822 see the whole document</p> <p>---</p> <p style="text-align: center;">-/--</p>	1-37

# INTERNATIONAL SEARCH REPORT

Intern: al Application No  
PCT/US 98/03179

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>M. SANICOLA ET AL.: "Gial cel line-derived neurotrophic factor-dependent RET activation can be mediated by two different cell-surface accessory proteins" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 94, no. 12, 10 June 1997, WASHINGTON US, pages 6238-6243, XP002065823 see abstract see page 6240, left-hand column, paragraph 2 - page 6243, left-hand column, paragraph 5</p>	<p>1-10, 16-19, 26-37</p>
P,X	<p>--- JING S ET AL: "GFRalpha-2 and GFRalpha-3 are two new receptors for ligands of the GDNF family." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 52, 26 December 1997, MD US, pages 33111-33117, XP002065824 see the whole document -----</p>	<p>1-37</p>

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 98/03179

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Although claims 20-23, as far as concerning an in-vivo method, are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compounds.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

### Information on patent family members

PCT/US 98/03179

09-12-1997